(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
20 February 2003 (20.02.2003)

PCT

(10) International Publication Number
WO 03/014330 A2

(51) International Patent Classification[7]: C12N 1/00

(21) International Application Number: PCT/EP02/08465

(22) International Filing Date: 30 July 2002 (30.07.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/309,877 6 August 2001 (06.08.2001) US

(71) Applicant *(for all designated States except US)*: **DEGUSSA AG** [DE/DE]; Bennigsenplatz 1, 40474 Düsseldorf (DE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **BATHE, Brigitte** [DE/DE]; Twieten 1, 33154 Salzkotten (DE). **KREUTZER, Caroline** [DE/DE]; Poststrasse 16, 49326 Melle (DE). **MÖCKEL, Bettina** [DE/DE]; Benrodestrasse 35, 40597 Düsseldorf (DE). **THIERBACH, Georg** [DE/DE]; Gunststrasse 21, 33613 Bielefeld (DE).

(74) Common Representative: **DEGUSSA AG**; Intellectual Property Management, Patents and Trademarks, Location Hanau, P. O. Box 13 45, 63403 Hanau (DE).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW, ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)*
— *of inventorship (Rule 4.17(iv)) for US only*

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/014330 A2

(54) Title: CORYNEFORM BACTERIA WHICH PRODUCE CHEMICAL COMPOUNDS II

(57) Abstract: The invention relates to coryneform bacteria, which instead of the singular copy of an open reading frame (ORF), gene or allele naturally present at the particular desired site (locus), have at least two copies of the open reading frame (ORF), gene or allele in question, preferably in tandem arrangement, and optionally at least a third copy of the open reading frame (ORF), gene or allele in question at a further gene site, and processes for the preparation of chemical compounds by fermentation of these bacteria.

Coryneform Bacteria which Produce Chemical Compounds II

Prior Art

Chemical compounds, which means, in particular, L-amino acids, vitamins, nucleosides and nucleotides and D-amino acids, are used in human medicine, in the pharmaceuticals industry, in cosmetics, in the foodstuffs industry and in animal nutrition.

Numerous of these compounds are prepared by fermentation from strains of coryneform bacteria, in particular Corynebacterium glutamicum. Because of their great importance, work is constantly being undertaken to improve the preparation processes. Improvements to the process can relate to fermentation measures, such as, for example, stirring and supply of oxygen, or the composition of the nutrient media, such as, for example, the sugar concentration during the fermentation, or the working up to the product form by, for example, ion exchange chromatography, or the intrinsic output properties of the microorganism itself.

Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these microorganisms. Strains which are resistant to antimetabolites or are auxotrophic for metabolites of regulatory importance and which produce the particular compounds are obtained in this manner.

Methods of the recombinant DNA technique have also been employed for some years for improving the strain of Corynebacterium strains, by amplifying individual biosynthesis genes and investigating the effect on production.

A common method comprises amplification of certain biosynthesis genes in the particular microorganism by means of episomally replicating plasmids. This procedure has the

disadvantage that during the fermentation, which in industrial processes is in general associated with numerous generations, the plasmids are lost spontaneously (segregational instability).

Another method comprises duplicating certain biosynthesis genes by means of plasmids which do not replicate in the particular microorganism. In this method, the plasmid, including the cloned biosynthesis gene, is integrated into the chromosomal biosynthesis gene of the microorganism (Reinscheid et al., Applied and Environmental Microbiology 60(1), 126-132 (1994); Jetten et al., Applied Microbiology and Biotechnology 43(1):76-82 (1995)). A disadvantage of this method is that the nucleotide sequences of the plasmid and of the antibiotic resistance gene necessary for the selection remain in the microorganism. This is a disadvantage, for example, for the disposal and utilization of the biomass. Moreover, the expert expects such strains to be unstable as a result of disintegration by "Campbell type cross over" in a corresponding number of generations such as are usual in industrial fermentations.

Object of the Invention

The inventors had the object of providing new measures for improved fermentative preparation of chemical compounds using coryneform bacteria.

Summary of the Invention

The invention provides coryneform bacteria, in particular of the genus Corynebacterium, which produce one or more desired chemical compounds, characterized in that

a) instead of the singular copy of an open reading frame (ORF), gene or allele naturally present at the particular desired site (locus), these have at least two copies of the said open reading frame (ORF), gene or allele, preferably in tandem arrangement, no

nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the particular site, and in that these

b) optionally have at least a third copy of the open reading frame (ORF), gene or allele in question at a further gene site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the further gene site.

The invention also provides processes for the preparation of one or more chemical compounds, which comprise the following steps:

a) fermentation of coryneform bacteria, in particular of the genus Corynebacterium, which

i) instead of the singular copy of an open reading frame (ORF), gene or allele naturally present at the particular desired site (locus), have at least two copies of the said open reading frame (ORF), gene or allele, preferably in tandem arrangement, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the particular site, and in that these

ii) optionally have at least a third copy of the said open reading frame (ORF), gene or allele at a

further gene site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the further gene site,

under conditions which allow expression of the said open reading frames (ORFs) genes or alleles,

b) concentration of the chemical compound(s) in the fermentation broth and/or in the cells of the bacteria,

c) isolation of the chemical compound(s), optionally

d) with constituents from the fermentation broth and/or the biomass to the extent of > (greater than) 0 to 100%.

Detailed Description of the Invention

Chemical compounds are to be understood, in particular, as meaning amino acids, vitamins, nucleosides and nucleotides. The biosynthesis pathways of these compounds are known and are available in the prior art.

Amino acids mean, preferably, L-amino acids, in particular the proteinogenic L-amino acids, chosen from the group consisting of L-aspartic acid, L-asparagine, L-threonine, L-serine, L-glutamic acid, L-glutamine, glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan, L-proline and L-arginine and salts thereof, in particular L-lysine, L-methionine and L-threonine. L-Lysine is very particularly preferred.

Proteinogenic amino acids are understood as meaning the amino acids which occur in natural proteins, that is to say in proteins of microorganisms, plants, animals and humans.

Vitamins mean, in particular, vitamin B1 (thiamine), vitamin B2 (riboflavin), vitamin B5 (pantothenic acid), vitamin B6 (pyridoxines), vitamin B12 (cyanocobalamin), nicotinic acid/nicotinamide, vitamin M (folic acid) and vitamin E (tocopherol) and salts thereof, pantothenic acid being preferred.

Nucleosides and nucleotides mean, inter alia, S-adenosyl-methionine, inosine-5'-monophosphoric acid and guanosine-5'-monophosphoric acid and salts thereof.

The coryneform bacteria are, in particular, those of the genus Corynebacterium. Of the genus Corynebacterium, the species Corynebacterium glutamicum, Corynebacterium ammoniagenes and Corynebacterium thermoaminogenes are preferred. Information on the taxonomic classification of strains of this group of bacteria is to be found, inter alia, in Kämpfer and Kroppenstedt (Canadian Journal of Microbiology 42, 989-1005 (1996)) and in US-A-5,250,434.

Suitable strains of the species Corynebacterium glutamicum (C. glutamicum) are, in particular, the known wild-type strains

Corynebacterium glutamicum ATCC13032
Corynebacterium acetoglutamicum ATCC15806
Corynebacterium acetoacidophilum ATCC13870
Corynebacterium lilium ATCC15990
Corynebacterium melassecola ATCC17965
Corynebacterium herculis ATCC13868
Arthrobacter sp ATCC243
Brevibacterium chang-fua ATCC14017
Brevibacterium flavum ATCC14067
Brevibacterium lactofermentum ATCC13869

Brevibacterium divaricatum ATCC14020
Brevibacterium taipei ATCC13744 and
Microbacterium ammoniaphilum ATCC21645

and mutants or strains, such as are known from the prior art, produced therefrom which produce chemical compounds.

Suitable strains of the species Corynebacterium ammoniagenes (C. ammoniagenes) are, in particular, the known wild-type strains

Brevibacterium ammoniagenes ATCC6871
Brevibacterium ammoniagenes ATCC15137 and
Corynebacterium sp. ATCC21084

and mutants or strains, such as are known from the prior art, produced therefrom which produce chemical compounds.

Suitable strains of the species Corynebacterium thermoaminogenes (C. thermoaminogenes) are, in particular, the known wild-type strains

Corynebacterium thermoaminogenes FERM BP-1539
Corynebacterium thermoaminogenes FERM BP-1540
Corynebacterium thermoaminogenes FERM BP-1541 and
Corynebacterium thermoaminogenes FERM BP-1542

and mutants or strains, such as are known from the prior art, produced therefrom which produce chemical compounds.

Strains with the designation "ATCC" can be obtained from the American Type Culture Collection (Manassas, VA, USA). Strains with the designation "FERM" can be obtained from the National Institute of Advanced Industrial Science and Technology (AIST Tsukuba Central 6, 1-1-1 Higashi, Tsukuba Ibaraki, Japan). The strains of Corynebacterium thermoaminogenes mentioned (FERM BP-1539, FERM BP-1540, FERM BP-1541 and FERM BP-1542) are described in US-A 5,250,434.

Open reading frame (ORF) describes a section of a nucleotide sequence which codes or can code for a protein or polypeptide or ribonucleic acid to which no function can be assigned according to the prior art.

After assignment of a function to the nucleotide sequence section in question, it is in general referred to as a gene.

Alleles are in general understood as meaning alternative forms of a given gene. The forms are distinguished by differences in the nucleotide sequence.

In the context of the present invention, endogenous, that is to say species-characteristic, open reading frames, genes or alleles are preferably used. These are understood as meaning the open reading frames, genes or alleles or nucleotide sequences thereof present in the population of a species, such as, for example, Corynebacterium glutamicum.

A "singular copy of an open reading frame (ORF), gene or allele naturally present at the particular desired site (locus)" is understood as meaning the circumstances that a gene in general naturally occurs in one (1) copy in the form of its nucleotide sequence at its site or gene site in the corresponding wild-type or corresponding parent organism or starting organism. This site is preferably in the chromosome.

Thus, for example, the lysC gene or an $lysC^{FBR}$ allele which codes for a "feed back" resistant aspartate kinase is present in one copy at the lysC site or lysC locus or lysC gene site and is flanked by the open reading frame orfX and the leuA gene on one side and by the asd gene on the other side.

"Feed back" resistant aspartokinases are understood as meaning aspartokinases which, compared with the wild-type form, have a lower sensitivity to inhibition by mixtures of

lysine and threonine or mixtures of AEC (aminoethylcysteine) and threonine or lysine by itself or AEC by itself. Strains which produce L-lysine typically contain such "feed back" resistant or desensitized aspartokinases.

The nucleotide sequence of the chromosome of Corynebacterium glutamicum is known and can be found in the patent application EP-A-1108790 and Access Number (Accession No.) AX114121 of the nucleotide sequence databank of the European Molecular Biologies Laboratories (EMBL, Heidelberg, Germany and Cambridge, UK). The nucleotide sequences of orfX, the leuA gene and the asd gene have the Access Numbers AX120364 (orfX), AX123517 (leuA) and AX123519 (asd).

Further databanks, such as, for example, that of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) or that of the Swiss Institute of Bioinformatics (Swissprot, Geneva, Switzerland) or that of the Protein Information Resource Database (PIR, Washington, DC, USA) can also be used.

"Tandem arrangement" of two or more copies of an open reading frame (ORF), gene or allele is referred to if these are arranged in a row directly adjacent in the same orientation.

"A further gene site" is understood as meaning a second gene site, the nucleotide sequence of which is different from the sequence of the ORF, gene or allele which has been at least duplicated at the natural site. This further gene site, or the nucleotide sequence present at the further gene site, is preferably in the chromosome and is in general not essential for growth and for production of the desired chemical compounds.

The "further gene sites" mentioned include, of course, not only the coding regions of the open reading frames or genes mentioned, but also the regions or nucleotide sequences lying upstream which are responsible for expression and regulation, such as, for example, ribosome binding sites, promoters, binding sites for regulatory proteins, binding sites for regulatory ribonucleic acids and attenuators. These regions in general lie in a range of 1-800, 1-600, 1-400, 1-200, 1-100 or 1-50 nucleotides upstream of the coding region. In the same way, regions lying downstream, such as, for example, transcription terminators, are also included. These regions in general lie in a range of 1-400, 1-200, 1-100, 1-50 or 1-25 nucleotides downstream of the coding region.

Intergenic regions in the chromosome, that is to say nucleotide sequences without a coding function, can furthermore be used. Finally, prophages or defective phages contained in the chromosome can be used for this.

A prophage is understood as meaning a bacteriophage, in particular the genome thereof, where this is replicated together with the genome of the host and the formation of infectious particles does not take place. A defective phage is understood as meaning a prophage, in particular the genome thereof, which, as a result of various mutations, has lost the ability to form so-called infectious particles. Defective phages are also called cryptic. Prophages and defective phages are often present in integrated form in the chromosome of their host. Further details exist in the prior art, for example in the textbook by Edward A. Birge (Bacterial and Bacteriophage Genetics, 3rd ed., Springer-Verlag, New York, USA, 1994) or in the textbook by S. Klaus et al. (Bakterienviren, Gustav Fischer Verlag, Jena, Germany, 1992).

To produce the coryneform bacteria according to the invention, the nucleotide sequence of the desired ORF, gene

or allele, preferably including the expression and/or regulation signals, is isolated, at least two copies are arranged in a row, preferably in tandem arrangement, these are then transferred into the desired coryneform bacterium, preferably with the aid of vectors which do not replicate or replicate to only a limited extent in coryneform bacteria, and those bacteria in which two copies of the ORF, gene or allele are incorporated at the particular desired natural site instead of the singular copy originally present are isolated, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remaining at the particular natural site (locus).

The expression and/or regulation signals mentioned, such as, for example, the ribosome binding sites, promoters, binding sites for regulatory proteins, binding sites for regulatory ribonucleic acids and attenuators lying upstream of the coding region of the ORF, gene or allele, are in general in a range of 1-800, 1-600, 1-400, 1-200, 1-100 or 1-50 nucleotides upstream of the coding region. The expression and/or regulation signals mentioned, such as, for example, the transcription terminators lying downstream of the coding region of the ORF, gene or allele, are in general in a range of 1-400, 1-200, 1-100, 1-50 or 1-25 nucleotides downstream of the coding region.

Preferably, also, no residues of sequences of the vectors used or species-foreign DNA, such as, for example, restriction cleavage sites, remain on the flanks of the ORFs, genes or alleles amplified according to the invention. In each case a maximum of 24, preferably a maximum of 12, particularly preferably a maximum of 6 nucleotides of such DNA optionally remain on the flanks.

At least a third copy of the open reading frame (ORF), gene or allele in question is optionally inserted at a further gene site, or several further gene sites, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the further gene site.

Preferably, also, no residues of sequences of the vectors used or species-foreign DNA, such as, for example, restriction cleavage sites, remain at the further gene site. A maximum of 24, preferably a maximum of 12, particularly preferably a maximum of 6 nucleotides of such DNA upstream or downstream of the ORF, gene or allele incorporated optionally remain at the further gene site.

The invention accordingly also provides a process for the production of coryneform bacteria which produce one or more chemical compounds, characterized in that

a) the nucleotide sequence of a desired ORF, gene or allele, preferably including the expression and/or regulation signals, is isolated

b) at least two copies of the nucleotide sequence of the ORF, gene or allele are arranged in a row, preferably in tandem arrangement

c) the nucleotide sequence obtained according to b) is incorporated in a vector which does not replicate or replicates to only a limited extent in coryneform bacteria,

d) the nucleotide sequence according to b) or c) is transferred into coryneform bacteria, and

e) coryneform bacteria which have at least two copies of the desired ORF, gene or allele at the particular

desired natural site instead of the singular copy of the ORF, gene or allele originally present are isolated, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remaining at the particular natural site (locus), and

f) at least a third copy of the open reading frame (ORF), gene or allele in question is optionally introduced at a further gene site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remaining at the further gene site.

By the measures according to the invention, the productivity of the coryneform bacteria or of the fermentative processes for the preparation of chemical compounds is improved in respect of one or more of the features chosen from the group consisting of concentration (chemical compound formed, based on the unit volume), yield (chemical compound formed, based on the source of carbon consumed) and product formation rate (chemical compound formed, based on the time) by at least 0.5 - 1.0% or at least 1.0 to 1.5% or at least 1.5 - 2.0%.

Instructions on conventional genetic engineering methods, such as, for example, isolation of chromosomal DNA, plasmid DNA, handling of restriction enzymes etc., are found in Sambrook et al. (Molecular Cloning - A Laboratory Manual (1989) Cold Spring Harbor Laboratory Press). Instructions on transformation and conjugation in coryneform bacteria are found, inter alia, in Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)), in Schäfer et al. (Journal of Bacteriology 172, 1663-1666

(1990) and Gene 145, 69-73 (1994)) and in Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)).

Vectors which replicate to only a limited extent are understood as meaning plasmid vectors which, as a function of the conditions under which the host or carrier is cultured, replicate or do not replicate. Thus, a temperature-sensitive plasmid for coryneform bacteria which can replicate only at temperatures below 31ºC has been described by Nakamura et al. (US-A-6,303,383).

The invention also provides coryneform bacteria, in particular of the genus Corynebacterium, which produce L-lysine, characterized in that

a) instead of the singular copy of an open reading frame (ORF), a gene or allele of lysine production naturally present at the particular desired site (locus), these have at least two copies of the said open reading frame (ORF), gene or allele, preferably in tandem arrangement, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the particular site, and in that these

b) optionally have at least a third copy of the said open reading frame (ORF), gene or allele of L-lysine production at a further gene site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the further gene site.

The invention also furthermore provides a process for the preparation of L-lysine, which comprises the following steps:

a) fermentation of coryneform bacteria, in particular of the genus Corynebacterium, which

i) instead of the singular copy of an open reading frame (ORF), gene or allele of lysine production present at the particular desired site (locus), have at least two copies of the open reading frame (ORF), gene or allele in question, preferably in tandem arrangement, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the particular site, and in that these

ii) optionally have at least a third copy of the open reading frame (ORF), gene or allele of L-lysine production in question at a further gene site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the further gene site,

under conditions which allow expression of the said open reading frames (ORFs), genes or alleles,

b) concentration of the L-lysine in the fermentation broth,

c) isolation of the L-lysine from the fermentation broth, optionally

d) with constituents from the fermentation broth and/or the biomass to the extent of > (greater than) 0 to 100%.

A "copy of an open reading frame (ORF), gene or allele of lysine production" is to be understood as meaning all the, preferably endogenous, open reading frames, genes or alleles of which enhancement/over-expression can have the effect of improving lysine production. Enhancement is understood as meaning an increase in the intracellular concentration or activity of the particular gene product, protein or enzyme.

These include, inter alia, the following open reading frames, genes or alleles: accBC, accDA, cstA, cysD, cysE, cysH, cysK, cysN, cysQ, dapA, dapB, dapC, dapD, dapE, dapF, ddh, dps, eno, gap, gap2, gdh, gnd, lysC, $lysC^{FBR}$, lysE, msiK, opcA, oxyR, ppc, ppc^{FBR}, pgk, pknA, pknB, pknD, pknG, ppsA, ptsH, ptsI, ptsM, pyc, pyc P458S, sigC, sigD, sigE, sigH, sigM, tal, thyA, tkt, tpi, zwa1, zwf and zwf A213T. These are summarized and explained in Table 1.

These include, in particular, the $lysC^{FBR}$ alleles which code for a "feed back" resistant aspartate kinase. Various $lysC^{FBR}$ alleles are summarized and are explained in Table 2.

The following $lysC^{FBR}$ alleles are preferred: lysC A279T (replacement of alanine at position 279 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by threonine), lysC A279V (replacement of alanine at position 279 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by valine), lysC S301F (replacement of serine at position 301 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by phenylalanine), lysC T308I (replacement of threonine at position 308 of the aspartate

kinase protein coded, according to SEQ ID NO: 2, by isoleucine), lysC S301Y (replacement of serine at position 308 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by tyrosine), lysC G345D (replacement of glycine at position 345 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by aspartic acid), lysC R320G (replacement of arginine at position 320 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by glycine), lysC T311I (replacement of threonine at position 311 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by isoleucine), lysC S381F (replacement of serine at position 381 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by phenylalanine).

The $lysC^{FBR}$ allele lysC T311I (replacement of threonine at position 311 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by isoleucine), the nucleotide sequence of which is shown as SEQ ID NO:3, is particularly preferred; the amino acid sequence of the aspartate kinase protein coded is shown as SEQ ID NO:4.

The following open reading frames, genes or nucleotide sequences, inter alia, can be used as the "further gene site" which is not essential for growth or lysine production: aecD, ccpA1, ccpA2, citA, citB, citE, fda, gluA, gluB, gluC, gluD, luxR, luxS, lysR1, lysR2, lysR3, menE, mqo, pck, pgi, poxB and zwa2, in particular the genes aecD, gluA, gluB, gluC, gluD and pck. These are summarized and explained in Table 3. Intergenic regions in the chromosome, that is to say nucleotide sequences without a coding function, can furthermore be used. Finally, prophages or defective phages contained in the chromosome can be used.

Table 1

Open reading frames, genes and alleles of lysine production

Name	Description of the coded enzyme or protein	Reference	Access Number
accBC	Acyl-CoA Carboxylase EC 6.3.4.14 (acyl-CoA carboxylase)	Jäger et al. Archives of Microbiology (1996) 166:76-82 EP1108790; WO0100805	U35023 AX123524 AX066441
accDA	Acetyl-CoA Carboxylase EC 6.4.1.2 (acetyl-CoA carboxylase)	EP1055725 EP1108790 WO0100805	 AX121013 AX066443
cstA	Carbon Starvation Protein A (carbon starvation protein A)	EP1108790 WO0100804	AX120811 AX066109
cysD	Sulfate Adenylyltransferase sub-unit II EC 2.7.7.4 (sulfate adenylyltransferase small chain)	EP1108790	AX123177
cysE	Serine Acetyltransferase EC 2.3.1.30 (serine acetyltransferase)	EP1108790 WO0100843	AX122902 AX063961
cysH	3'-Phosphoadenyl Sulfate Reductase EC 1.8.99.4 (3'-phosphoadenosine 5'-phosphosulfate reductase)	EP1108790 WO0100842	AX123178 AX066001
cysK	Cysteine Synthase EC 4.2.99.8 (cysteine synthase)	EP1108790 WO0100843	AX122901 AX063963
cysN	Sulfate Adenylyltransferase sub-unit I EC 2,7.7.4 (sulfate adenylyltransferase)	EP1108790	AX123176 AX127152
cysQ	Transport protein CysQ (transporter cysQ)	EP1108790 WO0100805	AX127145 AX066423
dapA	Dihydrodipicolinate Synthase EC 4.2.1.52 (dihydrodipicolinate synthase)	Bonnassie et al. Nucleic Acids Research 18:6421 (1990) Pisabarro et al., Journal of Bacteriology 175:2743-2749(1993) EP1108790 WO0100805 EP0435132 EP1067192 EP1067193	X53993 Z21502 AX123560 AX063773
dapB	Dihydrodipicolinate Reductase EC 1.3.1.26 (dihydrodipicolinate reductase)	EP1108790 WO0100843 EP1067192 EP1067193	AX127149 AX063753 AX137723 AX137602

		Pisabarro et al., Journal of Bacteriology 175:2743-2749(1993) JP1998215883 JP1997322774 JP1997070291 JP1995075578	X67737 Z21502 E16749 E14520 E12773 E08900
dapC	N-Succinyl Aminoketopimelate Transaminase EC 2.6.1.17 (N-succinyl diaminopimelate transaminase)	EP1108790 WO0100843 EP1136559	AX127146 AX064219
dapD	Tetrahydrodipicolinate Succinylase EC 2.3.1.117 (tetrahydrodipicolinate succinylase)	EP1108790 WO0100843 Wehrmann et al. Journal of Bacteriology 180:3159-3165(1998)	AX127146 AX063757 AJ004934
dapE	N-Succinyl Diaminopimelate Desuccinylase EC 3.5.1.18 (N-succinyl diaminopimelate desuccinylase)	EP1108790 WO0100843 Wehrmann et al. Microbiology 140:3349-3356 (1994)	AX127146 AX063749 X81379
dapF	Diaminopimelate Epimerase EC 5.1.1.7 (diaminopimelate epimerase)	EP1108790 WO0100843 EP1085094	AX127149 AX063719 AX137620
ddh	Diaminopimelate Dehydrogenase EC 1.4.1.16 (diaminopimelate dehydrogenase)	EP1108790 WO0100843 Ishino et al., Nucleic Acids Research 15:3917-3917(1987) JP1997322774 JP1993284970 Kim et al., Journal of Microbiology and Biotechnology 5:250-256(1995)	AX127152 AX063759 Y00151 E14511 E05776 D87976
dps	DNA Protection Protein (protection during starvation protein)	EP1108790	AX127153
eno	Enolase EC 4.2.1.11 (enolase)	EP1108790 WO0100844 EP1090998 Hermann et al., Electrophoresis 19:3217-3221 (1998)	AX127146 AX064945 AX136862
gap	Glyceraldehyde 3-Phosphate Dehydrogenase EC 1.2.1.12	EP1108790 WO0100844 Eikmanns et	AX127148 AX064941 X59403

	(glyceraldehyde 3-phosphate dehydrogenase)	al., Journal of Bacteriology 174:6076-6086(1992)	
gap2	Glyceraldehyde 3-Phosphate Dehydrogenase EC 1.2.1.12 (glyceraldehyde 3-phosphate dehydrogenase 2)	EP1108790 WO0100844	AX127146 AX064939
gdh	Glutamate Dehydrogenase EC 1.4.1.4 (glutamate dehydrogenase)	EP1108790 WO0100844 Boermann et al., Molecular Microbiology 6:317-326 (1992). Guyonvarch et al. NCBI	AX127150 AX063811 X59404 X72855
gnd	6-Phosphogluconate Dehydrogenase EC 1.1.1.44 (6-phosphogluconate dehydrogenase)	EP1108790 WO0100844	AX127147 AX121689 AX065125
lysC	Aspartate Kinase EC 2.7.2.4 (aspartate kinase)	EP1108790 WO0100844 Kalinowski et al., Molecular Microbiology 5:1197-204 (1991)	AX120365 AX063743 X57226
$lysC^{FBR}$	Aspartate Kinase feedback resistent (fbr) EC 2.7.2.4 (aspartate kinase fbr)	see Table 2	
lysE	Lysine Exporter (lysine exporter protein)	EP1108790 WO0100843 Vrljić et al., Molecular Microbiology 22:815-826 (1996)	AX123539 AX123539 X96471
msiK	Sugar Importer (multiple sugar import protein)	EP1108790	AX120892
opcA	Glucose 6-Phosphate Dehydrogenase (subunit of glucose 6-phosphate dehydrogenase)	WO0104325	AX076272
oxyR	Transcription Regulator (transcriptional regulator)	EP1108790	AX122198 AX127149
ppc^{FBR}	Phosphoenol Pyruvate Carboxylase feedback resistent EC 4.1.1.31 (phosphoenol pyruvate carboxylase feedback resistant)	EP0723011 WO0100852	
ppc	Phosphoenol Pyruvate Carboxylase EC 4.1.1.31 (phosphoenol pyruvate carboxylase)	EP1108790 O'Reagan et al., Gene 77(2):237-251(1989)	AX127148 AX123554 M25819
pgk	Phosphoglycerate Kinase	EP1108790	AX121838

	EC 2.7.2.3 (phosphoglycerate kinase)	WO0100844 Eikmanns, Journal of Bacteriology 174:6076-6086 (1992)	AX127148 AX064943 X59403
pknA	Protein Kinase A (protein kinase A)	EP1108790	AX120131 AX120085
pknB	Protein Kinase B (protein kinase B)	EP1108790	AX120130 AX120085
pknD	Protein Kinase D (protein kinase D)	EP1108790	AX127150 AX122469 AX122468
pknG	Protein Kinase G (protein kinase G)	EP1108790	AX127152 AX123109
ppsA	Phosphoenol Pyruvate Synthase EC 2.7.9.2 (phosphoenol pyruvate synthase)	EP1108790	AX127144 AX120700 AX122469
ptsH	Phosphotransferase System Protein H EC 2.7.1.69 (phosphotransferase system component H)	EP1108790 WO0100844	AX122210 AX127149 AX069154
ptsI	Phosphotransferase System Enzyme I EC 2.7.3.9 (phosphotransferase system enzyme I)	EP1108790	AX122206 AX127149
ptsM	Glucose-specific Phosphotransferase System Enzyme II EC 2.7.1.69 (glucose phosphotransferase-system enzyme II)	Lee et al., FEMS Microbiology Letters 119(1-2):137-145 (1994)	L18874
pyc	Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase)	WO9918228 Peters-Wendisch et al., Microbiology 144:915-927 (1998)	A97276 Y09548
pyc P458S	Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) amino acid exchange P458S	EP1108790	
sigC	Sigma Factor C EC 2.7.7.6 (extracytoplasmic function alternative sigma factor C)	EP1108790	AX120368 AX120085
sigD	RNA Polymerase Sigma Factor D EC 2.7.7.6 (RNA polymerase sigma factor)	EP1108790	AX120753 AX127144
sigE	Sigma Factor E EC 2.7.7.6 (extracytoplasmic function alternative sigma factor E)	EP1108790	AX127146 AX121325
sigH	Sigma Factor H EC 2.7.7.6 (sigma factor SigH)	EP1108790	AX127145 AX120939

sigM	Sigma Factor M EC 2.7.7.6 (sigma factor SigM)	EP1108790	AX123500 AX127153
tal	Transaldolase EC 2.2.1.2 (transaldolase)	WO0104325	AX076272
thyA	Thymidylate Synthase EC 2.1.1.45 (thymidylate synthase)	EP1108790	AX121026 AX127145
tkt	Transketolase EC 2.2.1.1 (transketolase)	Ikeda et al., NCBI	AB023377
tpi	Triose Phosphate Isomerase EC 5.3.1.1 (triose phosphate isomerase)	Eikmanns, Journal of Bacteriology 174:6076-6086 (1992)	X59403
zwa1	Cell Growth Factor 1 (growth factor 1)	EP1111062	AX133781
zwf	Glucose 6-Phosphate 1-Dehydrogenase EC 1.1.1.49 (glucose 6-phosphate 1-dehydrogenase)	EP1108790 WO0104325	AX127148 AX121827 AX076272
zwf A213T	Glucose 6-Phosphate 1-Dehydrogenase EC 1.1.1.49 (glucose 6-phosphate 1-dehydrogenase) amino acid exchange A213T	EP1108790	

Table 2

$lysC^{FBR}$ alleles which code for feed back resistant aspartate kinases

Name of the allele	Amino acid replacement	Reference	Access Number
$lysC^{FBR}$-E05108		JP 1993184366-A (sequence 1)	E05108
$lysC^{FBR}$-E06825	lysC A279T	JP 1994062866-A (sequence 1)	E06825
$lysC^{FBR}$-E06826	lysC A279T	JP 1994062866-A (sequence 2)	E06826
$lysC^{FBR}$-E06827		JP 1994062866-A (sequence 3)	E06827
$lysC^{FBR}$-E08177		JP 1994261766-A (sequence 1)	E08177
$lysC^{FBR}$-E08178	lysC A279T	JP 1994261766-A (sequence 2)	E08178
$lysC^{FBR}$-E08179	lysC A279V	JP 1994261766-A (sequence 3)	E08179
$lysC^{FBR}$-E08180	lysC S301F	JP 1994261766-A (sequence 4)	E08180
$lysC^{FBR}$-E08181	lysC T308I	JP 1994261766-A (sequence 5)	E08181
$lysC^{FBR}$-E08182		JP 1994261766-A	E08182
$lysC^{FBR}$-E12770		JP 1997070291-A (sequence 13)	E12770
$lysC^{FBR}$-E14514		JP 1997322774-A (sequence 9)	E14514
$lysC^{FBR}$-E16352		JP 1998165180-A (sequence 3)	E16352
$lysC^{FBR}$-E16745		JP 1998215883-A (sequence 3)	E16745
$lysC^{FBR}$-E16746		JP 1998215883-A (sequence 4)	E16746
$lysC^{FBR}$-I74588		US 5688671-A (sequence 1)	I74588
$lysC^{FBR}$-I74589	lysC A279T	US 5688671-A (sequence 2)	I74589
$lysC^{FBR}$-I74590		US 5688671-A (sequence 7)	I74590
$lysC^{FBR}$-I74591	lysC A279T	US 5688671-A (sequence 8)	I74591
$lysC^{FBR}$-I74592		US 5688671-A (sequence 9)	I74592

$lysC^{FBR}$-I74593	lysC A279T	US 5688671-A (sequence 10)	I74593
$lysC^{FBR}$-I74594		US 5688671-A (sequence 11)	I74594
$lysC^{FBR}$-I74595	lysC A279T	US 5688671-A (sequence 12)	I74595
$lysC^{FBR}$-I74596		US 5688671-A (sequence 13)	I74596
$lysC^{FBR}$-I74597	lysC A279T	US 5688671-A (sequence 14)	I74597
$lysC^{FBR}$-X57226	lysC S301Y	EP0387527 Kalinowski et al., Molecular and General Genetics 224:317-324 (1990)	X57226
$lysC^{FBR}$-L16848	lysC G345D	Follettie and Sinskey NCBI Nucleotide Database (1990)	L16848
$lysC^{FBR}$-L27125	lysC R320G lysC G345D	Jetten et al., Applied Microbiology Biotechnology 43:76-82 (1995)	L27125
$lysC^{FBR}$	lysC T311I	WO0063388 (sequence 17)	
$lysC^{FBR}$	lysC S301F	US3732144	
$lysC^{FBR}$	lysC S381F		
$lysC^{FBR}$		JP6261766 (sequence 1)	
$lysC^{FBR}$	lysC A279T	JP6261766 (sequence 2)	
$lysC^{FBR}$	lysC A279V	JP6261766 (sequence 3)	
$lysC^{FBR}$	lysC S301F	JP6261766 (sequence 4)	
$lysC^{FBR}$	lysC T308I	JP6261766 (sequence 5)	

Table 3

Further gene sites for integration of open reading frames, genes and alleles of lysine production

Gene name	Description of the coded enzyme or protein	Reference	Access Number
aecD	beta C-S Lyase EC 2.6.1.1 (beta C-S lyase)	Rossol et al., Journal of Bacteriology 174(9):2968-77 (1992)	M89931
ccpA1	Catabolite Control Protein (catabolite control protein A1)	WO0100844 EP1108790	AX065267 AX127147
ccpA2	Catabolite Control Protein (catabolite control protein A2)	WO0100844 EP1108790	AX065267 AX121594
citA	Sensor Kinase CitA (sensor kinase CitA)	EP1108790	AX120161
citB	Transcription Regulator CitB (transcription regulator CitB)	EP1108790	AX120163
citE	Citrate Lyase EC 4.1.3.6 (citrate lyase)	WO0100844 EP1108790	AX065421 AX127146
fda	Fructose Bisphosphate Aldolase EC 4.1.2.13 (fructose 1,6-bisphosphate aldolase)	von der Osten et al., Molecular Microbiology 3(11):1625-37 (1989)	X17313
gluA	Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding protein)	Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)	X81191
gluB	Glutamate-binding Protein (glutamate-binding	Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)	X81191

	protein)		
gluC	Glutamate Transport Permease (glutamate transport system permease)	Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)	X81191
gluD	Glutamate Transport Permease (glutamate transport system permease)	Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)	X81191
luxR	Transcription Regulator LuxR (transcription regulator LuxR)	WO0100842 EP1108790	AX065953 AX123320
luxS	Histidine Kinase LuxS (histidine kinase LuxS)	EP1108790	AX123323 AX127153
lysR1	Transcription Regulator LysR1 (transcription regulator LysR1)	EP1108790	AX064673 AX127144
lysR2	Transcription Activator LysR2 (transcription regulator LysR2)	EP1108790	AX123312
lysR3	Transcription Regulator LysR3 (transcription regulator LysR3)	WO0100842 EP1108790	AX065957 AX127150
menE	O-Succinylbenzoic Acid CoA Ligase EC 6.2.1.26 (O-succinylbenzoate CoA ligase)	WO0100843 EP1108790	AX064599 AX064193 AX127144
mqo	Malate-Quinone Oxidoreductase (malate-quinone-oxidoreductase)	Molenaar et al., Eur. Journal of Biochemistry 1;254(2):395-403 (1998)	AJ224946
pck	Phosphoenol Pyruvate Carboxykinase (phosphoenol pyruvate	WO0100844	AJ269506 AX065053

	carboxykinase)		
pgi	Glucose 6-Phosphate Isomerase EC 5.3.1.9 (glucose-6-phosphate isomerase)	EP1087015 EP1108790	AX136015 AX127146
poxB	Pyruvate Oxidase EC 1.2.3.3 (pyruvate oxidase)	WO0100844 EP1096013	AX064959 AX137665
zwa2	Cell Growth Factor 2 (growth factor 2)	EP1106693 EP1108790	AX113822 AX127146

The invention accordingly also provides a process for the production of coryneform bacteria which produce L-lysine, characterized in that

a) the nucleotide sequence of a desired ORF, gene or allele of lysine production, optionally including the expression and/or regulation signals, is isolated

b) at least two copies of the nucleotide sequence of the ORF, gene or allele of lysine production are arranged in a row, preferably in tandem arrangement

c) the nucleotide sequence obtained according to b) is incorporated in a vector which does not replicate or replicates to only a limited extent in coryneform bacteria,

d) the nucleotide sequence according to b) or c) is transferred into coryneform bacteria, and

e) coryneform bacteria which have at least two copies of the desired ORF, gene or allele of lysine production at the particular desired natural site instead of the singular copy of the ORF, gene or allele originally present are isolated, no nucleotide sequence which is capable of/enables episomal replication in

microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remaining at the particular natural site (locus), and optionally

f) at least a third copy of the open reading frame (ORF), gene or allele of lysine production in question is introduced at a further gene site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remaining at the further gene site.

The invention also provides coryneform bacteria, in particular of the genus Corynebacterium, which produce L-methionine and/or L-threonine, characterized in that

a) instead of the singular copy of an open reading frame (ORF), a gene or allele of methionine production or threonine production naturally present at the particular desired site (locus), these have at least two copies of the said open reading frame (ORF), gene or allele, preferably in tandem arrangement, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the particular site, and in that these

b) optionally have at least a third copy of the open reading frame (ORF), gene or allele of methionine production or threonine production mentioned at a further gene site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is

capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the further gene site.

The invention also furthermore provides a process for the preparation of L-methionine and/or L-threonine, which comprises the following steps:

a) fermentation of coryneform bacteria, in particular of the genus Corynebacterium, which

i) instead of the singular copy of an open reading frame (ORF), gene or allele of methionine production or threonine production present at the particular desired site (locus), have at least two copies of the open reading frame (ORF), gene or allele in question, preferably in tandem arrangement, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the particular site, and

ii) optionally have at least a third copy of the open reading frame (ORF), gene or allele of methionine production or threonine production in question at a further gene site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the further gene site,

under conditions which allow expression of the

said open reading frames (ORFs), genes or alleles,

b) concentration of the L-methionine and/or L-threonine in the fermentation broth,

c) isolation of the L-methionine and/or L-threonine from the fermentation broth, optionally

d) with constituents from the fermentation broth and/or the biomass to the extent of > (greater than) 0 to 100%.

A "copy of an open reading frame (ORF), gene or allele of methionine production" is to be understood as meaning all the, preferably endogenous, open reading frames, genes or alleles of which enhancement/over-expression can have the effect of improving methionine production.

These include, inter alia, the following open reading frames, genes or alleles: accBC, accDA, aecD, cstA, cysD, cysE, cysH, cysK, cysN, cysQ, dps, eno, fda, gap, gap2, gdh, gnd, glyA, hom, hom^{FBR}, lysC, $lysC^{FBR}$, metA, metB, metE, metH, metY, msiK, opcA, oxyR, ppc, ppc^{FBR}, pgk, pknA, pknB, pknD, pknG, ppsA, ptsH, ptsI, ptsM, pyc, pyc P458S, sigC, sigD, sigE, sigH, sigM, tal, thyA, tkt, tpi, zwa1, zwf and zwf A213T. These are summarized and explained in Table 4. These include, in particular, the $lysC^{FBR}$ alleles which code for a "feed back" resistant aspartate kinase (see Table 2) and the hom^{FBR} alleles which code for a "feed back" resistant homoserine dehydrogenase.

The at least third, optionally fourth or fifth copy of the open reading frame (ORF), gene or allele of methionine production in question can be integrated at a further site. The following open reading frames, genes or nucleotide sequences, inter alia, can be used for this: brnE, brnF, brnQ, ccpA1, ccpA2, citA, citB, citE, ddh, gluA, gluB, gluC, gluD, luxR, luxS, lysR1, lysR2, lysR3, menE, metD,

metK, pck, pgi, poxB and zwa2. These are summarized and explained in Table 5. Intergenic regions in the chromosome, that is to say nucleotide sequences without a coding function, can furthermore be used. Finally, prophages or defective phages contained in the chromosome can be used for this.

Table 4

Open reading frames, genes and alleles of methionine production

Name	Description of the coded enzyme or protein	Reference	Access Number
accBC	Acyl-CoA Carboxylase EC 6.3.4.14 (acyl-CoA carboxylase)	Jäger et al. Archives of Microbiology (1996) 166:76-82 EP1108790; WO0100805	U35023 AX123524 AX066441
accDA	Acetyl-CoA Carboxylase EC 6.4.1.2 (acetyl-CoA carboxylase)	EP1055725 EP1108790 WO0100805	 AX121013 AX066443
aecD	Cystathionine beta-Lyase EC 4.4.1.8 (cystathionine beta-lyase)	Rossol et al., Journal of Bacteriology 174:2968-2977 (1992)	M89931
cstA	Carbon Starvation Protein A (carbon starvation protein A)	EP1108790 WO0100804	AX120811 AX066109
cysD	Sulfate Adenylyltransferase sub-unit II EC 2.7.7.4 (sulfate adenylyltransferase small chain)	EP1108790	AX123177
cysE	Serine Acetyltransferase EC 2.3.1.30 (serine acetyltransferase)	EP1108790 WO0100843	AX122902 AX063961
cysH	3'-Phosphoadenyl Sulfate Reductase EC 1.8.99.4 (3'-phosphoadenosine 5'-phosphosulfate reductase)	EP1108790 WO0100842	AX123178 AX066001
cysK	Cysteine Synthase EC 4.2.99.8 (cysteine synthase)	EP1108790 WO0100843	AX122901 AX063963
cysN	Sulfate Adenylyltransferase sub-unit I EC 2.7.7.4 (sulfate adenylyltransferase)	EP1108790	AX123176 AX127152
cysQ	Transport protein CysQ (transporter cysQ)	EP1108790 WO0100805	AX127145 AX066423
dps	DNA Protection Protein (protection during starvation protein)	EP1108790	AX127153
eno	Enolase EC 4.2.1.11 (enolase)	EP1108790 WO0100844 EP1090998 Hermann et al., Electrophoresis 19:3217-3221 (1998)	AX127146 AX064945 AX136862
fda	Fructose Bisphosphate Aldolase EC 4.1.12.13	van der Osten et al., Molecular	X17313

	(fructose bisphosphate aldolase)	Microbiology 3:1625-1637 (1989)	
gap	Glyceraldehyde 3-Phosphate Dehydrogenase EC 1.2.1.12 (glyceraldehyde 3-phosphate dehydrogenase)	EP1108790 WO0100844 Eikmanns et al., Journal of Bacteriology 174:6076-6086(1992)	AX127148 AX064941 X59403
gap2	Glyceraldehyde 3-Phosphate Dehydrogenase EC 1.2.1.12 (glyceraldehyde 3-phosphate dehydrogenase 2)	EP1108790 WO0100844	AX127146 AX064939
gdh	Glutamate Dehydrogenase EC 1.4.1.4 (glutamate dehydrogenase)	EP1108790 WO0100844 Boermann et al., Molecular Microbiology 6:317-326 (1992) Guyonvarch et al, NCBI	AX127150 AX063811 X59404 X72855
glyA	Glycine/Serine Hydroxymethyltransferase EC 2.1.2.1 (glycine/serine hydroxymethyltransferase)	EP1108790	AX127146 AX121194
gnd	6-Phosphogluconate Dehydrogenase EC 1.1.1.44 (6-phosphogluconate dehydrogenase)	EP1108790 WO0100844	AX127147 AX121689 AX065125
hom	Homoserine Dehydrogenase EC 1.1.1.3 (homoserine dehydrogenase)	Peoples et al., Molecular Microbiology 2:63-72 (1988)	Y00546
hom^{FBR}	Homoserine Dehydrogenase feedback resistant (fbr) EC 1.1.1.3 (homoserine dehydrogenase fbr)	Reinscheid et al., Journal of Bacteriology 173:3228-30 (1991)	
lysC	Aspartate Kinase EC 2.7.2.4 (aspartate kinase)	EP1108790 WO0100844 Kalinowski et al., Molecular Microbiology 5:1197-204 (1991)	AX120365 AX063743 X57226
$lysC^{FBR}$	Aspartate Kinase feedback resistent (fbr) EC 2.7.2.4 (aspartate kinase fbr)	see Table 2	
metA	Homoserine Acetyltransferase EC 2.3.1.31 (homoserine acetyltransferase)	Park et al., Molecular Cells 8:286-94 (1998)	AF052652
metB	Cystathionine γ-Lyase EC 4.4.1.1 (cystathionine gamma-synthase)	Hwang et al., Molecular Cells 9:300-308 (1999)	AF126953
metE	Homocysteine Methyltransferase EC 2.1.1.14	EP1108790	AX127146 AX121345

	(homocysteine methyltransferase)		
metH	Homocysteine Methyltransferase (Vitamin B12-dependent) EC 2.1.1.14 (homocysteine methyltransferase)	EP1108790	AX127148 AX121747
metY	Acetylhomoserine Sulfhydrolase (acetylhomoserine sulfhydrolase)	EP1108790	AX120810 AX127145
msiK	Sugar Importer (multiple sugar import protein)	EP1108790	AX120892
opcA	Glucose 6-Phosphate Dehydrogenase (subunit of glucose 6-phosphate dehydrogenase)	WO0104325	AX076272
oxyR	Transcription Regulator (transcriptional regulator)	EP1108790	AX122198 AX127149
ppc^{FBR}	Phosphoenol Pyruvate Carboxylase feedback resistent EC 4.1.1.31 (phosphoenol pyruvate carboxylase feedback resistant)	EP0723011 WO0100852	
ppc	Phosphoenol Pyruvate Carboxylase EC 4.1.1.31 (phosphoenol pyruvate carboxylase)	EP1108790 O'Reagan et al., Gene 77(2):237-251(1989)	AX127148 AX123554 M25819
pgk	Phosphoglycerate Kinase EC 2.7.2.3 (phosphoglycerate kinase)	EP1108790 WO0100844 Eikmanns, Journal of Bacteriology 174:6076-6086 (1992)	AX121838 AX127148 AX064943 X59403
pknA	Protein Kinase A (protein kinase A)	EP1108790	AX120131 AX120085
pknB	Protein Kinase B (protein kinase B)	EP1108790	AX120130 AX120085
pknD	Protein Kinase D (protein kinase D)	EP1108790	AX127150 AX122469 AX122468
pknG	Protein Kinase G (protein kinase G)	EP1108790	AX127152 AX123109
ppsA	Phosphoenol Pyruvate Synthase EC 2.7.9.2 (phosphoenol pyruvate synthase)	EP1108790	AX127144 AX120700 AX122469
ptsH	Phosphotransferase System Protein H EC 2.7.1.69 (phosphotransferase system component H)	EP1108790 WO0100844	AX122210 AX127149 AX069154
ptsI	Phosphotransferase System Enzyme I EC 2.7.3.9 (phosphotransferase system enzyme I)	EP1108790	AX122206 AX127149
ptsM	Glucose-specific Phosphotransferase System Enzyme II EC 2.7.1.69 (glucose phosphotransferase-system enzyme II)	Lee et al., FEMS Microbiology Letters 119(1-2):137-145 (1994)	L18874

pyc	Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase)	WO9918228 Peters-Wendisch et al., Microbiology 144:915-927 (1998)	A97276 Y09548
pyc P458S	Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) amino acid exchange P458S	EP1108790	
sigC	Sigma Factor C EC 2.7.7.6 (extracytoplasmic function alternative sigma factor C)	EP1108790	AX120368 AX120085
sigD	RNA Polymerase Sigma Factor D EC 2.7.7.6 (RNA polymerase sigma factor)	EP1108790	AX120753 AX127144
sigE	Sigma Factor E EC 2.7.7.6 (extracytoplasmic function alternative sigma factor E)	EP1108790	AX127146 AX121325
sigH	Sigma Factor H EC 2.7.7.6 (sigma factor SigH)	EP1108790	AX127145 AX120939
sigM	Sigma Factor M EC 2.7.7.6 (sigma factor SigM)	EP1108790	AX123500 AX127153
tal	Transaldolase EC 2.2.1.2 (transaldolase)	WO0104325	AX076272
thyA	Thymidylate Synthase EC 2.1.1.45 (thymidylate synthase)	EP1108790	AX121026 AX127145
tkt	Transketolase EC 2.2.1.1 (transketolase)	Ikeda et al., NCBI	AB023377
tpi	Triose Phosphate Isomerase EC 5.3.1.1 (triose phosphate isomerase)	Eikmanns, Journal of Bacteriology 174:6076-6086 (1992)	X59403
zwa1	Cell Growth Factor 1 (growth factor 1)	EP1111062	AX133781
zwf	Glucose 6-Phosphate 1-Dehydrogenase EC 1.1.1.49 (glucose 6-phosphate 1-dehydrogenase)	EP1108790 WO0104325	AX127148 AX121827 AX076272
zwf A213T	Glucose 6-Phosphate 1-Dehydrogenase EC 1.1.1.49 (glucose 6-phosphate 1-dehydrogenase) amino acid exchange A213T	EP1108790	

Table 5

Further gene sites for integration of open reading frames, genes and alleles of methionine production

Gene name	Description of the coded enzyme or protein	Reference	Access Number
brnE	Transporter of branched-chain amino acids (branched-chain amino acid transporter)	EP1096010	AX137709 AX137714
brnF	Transporter of branched-chain amino acids (branched-chain amino acid transporter)	EP1096010	AX137709 AX137714
brnQ	Carrier protein of branched-chain amino acids (branched-chain amino acid transport system carrier protein)	Tauch et al., Archives of Microbiology 169(4):303-12 (1998) WO0100805 EP1108790	M89931 AX066841 AX127150
ccpA1	Catabolite Control Protein (catabolite control protein A1)	WO0100844 EP1108790	AX065267 AX127147
ccpA2	Catabolite Control Protein (catabolite control protein A2)	WO0100844 EP1108790	AX065267 AX121594
citA	Sensor Kinase CitA (sensor kinase CitA)	EP1108790	AX120161
citB	Transcription Regulator CitB (transcription regulator CitB)	EP1108790	AX120163
citE	Citrate Lyase EC 4.1.3.6 (citrate lyase)	WO0100844 EP1108790	AX065421 AX127146
ddh	Diaminopimelate Dehydrogenase EC 1.4.1.16 (diaminopimelate dehydrogenase)	Ishino et al., Nucleic Acids Research 15: 3917 (1987) EP1108790	S07384 AX127152
gluA	Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding protein)	Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)	X81191
gluB	Glutamate-binding Protein (glutamate-binding protein)	Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)	X81191
gluC	Glutamate Transport Permease	Kronemeyer et al., Journal of Bacteriology	X81191

	(glutamate transport system permease)	177(5):1152-8 (1995)	
gluD	Glutamate Transport Permease (glutamate transport system permease)	Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)	X81191
luxR	Transcription Regulator LuxR (transcription regulator LuxR)	WO0100842 EP1108790	AX065953 AX123320
luxS	Histidine Kinase LuxS (histidine kinase LuxS)	EP1108790	AX123323 AX127153
lysR1	Transcription Regulator LysR1 (transcription regulator LysR1)	EP1108790	AX064673 AX127144
lysR2	Transcription Activator LysR2 (transcription regulator LysR2)	EP1108790	AX123312
lysR3	Transcription Regulator LysR3 (transcription regulator LysR3)	WO0100842 EP1108790	AX065957 AX127150
menE	O-Succinylbenzoic Acid CoA Ligase EC 6.2.1.26 (O-succinylbenzoate CoA ligase)	WO0100843 EP1108790	AX064599 AX064193 AX127144
metD	Transcription Regulator MetD (transcription regulator MetD)	EP1108790	AX123327 AX127153
metK	Methionine Adenosyl Transferase EC 2.5.1.6 (S-adenosylmethionine synthetase)	WO0100843 EP1108790	AX063959 AX127148
pck	Phosphoenol Pyruvate Carboxykinase (phosphoenol pyruvate carboxykinase)	WO0100844	AJ269506 AX065053
pgi	Glucose 6-Phosphate Isomerase EC 5.3.1.9 (glucose-6-phosphate isomerase)	EP1087015 EP1108790	AX136015 AX127146
poxB	Pyruvate Oxidase EC 1.2.3.3 (pyruvate oxidase)	WO0100844 EP1096013	AX064959 AX137665
zwa2	Cell Growth Factor 2 (growth factor 2)	EP1106693 EP1108790	AX113822 AX127146

A "copy of an open reading frame (ORF), gene or allele of threonine production" is to be understood as meaning all the, preferably endogenous, open reading frames, genes or alleles of which enhancement/over-expression can have the effect of improving threonine production.

These include, inter alia, the following open reading frames, genes or alleles: accBC, accDA, cstA, cysD, cysE, cysH, cysI, cysN, cysQ, dps, eno, fda, gap, gap2, gdh, gnd, hom, hom^{FBR}, lysC, $lysC^{FBR}$, msiK, opcA, oxyR, ppc, ppc^{FBR}, pgk, pknA, pknB, pknD, pknG, ppsA, ptsH, ptsI, ptsM, pyc, pyc P458S, sigC, sigD, sigE, sigH, sigM, tal, thyA, tkt, tpi, thrB, thrC, thrE, zwa1, zwf and zwf A213T. These are summarized and explained in Table 6. These include, in particular, the $lysC^{FBR}$ alleles which code for a "feed back" resistant aspartate kinase (See Table 2) and the hom^{FBR} alleles which code for a "feed back" resistant homoserine dehydrogenase.

The at least third, optionally fourth or fifth copy of the open reading frame (ORF), gene or allele of threonine production in question can be integrated at a site. The following open reading frames, genes or nucleotide sequences, inter alia, can be used for this: ccpA1, ccpA2, citA, citB, citE, ddh, gluA, gluB, gluC, gluD, glyA, ilvA, ilvBN, ilvC, ilvD, luxR, luxS, lysR1, lysR2, lysR3, mdh, menE, metA, metD, pck, poxB, sigB and zwa2. These are summarized and explained in Table 7. Intergenic regions in the chromosome, that is to say nucleotide sequences without a coding function, can furthermore be used. Finally, prophages or defective phages contained in the chromosome can be used for this.

Table 6

Open reading frames, genes and alleles of threonine production

Name	Description of the coded enzyme or protein	Reference	Access Number
accBC	Acyl-CoA Carboxylase EC 6.3.4.14 (acyl-CoA carboxylase)	Jäger et al. Archives of Microbiology (1996) 166:76-82 EP1108790; WO0100805	U35023 AX123524 AX066441
accDA	Acetyl-CoA Carboxylase EC 6.4.1.2 (acetyl-CoA carboxylase)	EP1055725 EP1108790 WO0100805	 AX121013 AX066443
cstA	Carbon Starvation Protein A (carbon starvation protein A)	EP1108790 WO0100804	AX120811 AX066109
cysD	Sulfate Adenylyltransferase sub-unit II EC 2.7.7.4 (sulfate adenylyltransferase small chain)	EP1108790	AX123177
cysE	Serine Acetyltransferase EC 2.3.1.30 (serine acetyltransferase)	EP1108790 WO0100843	AX122902 AX063961
cysH	3'-Phosphoadenyl Sulfate Reductase EC 1.8.99.4 (3'-phosphoadenosine 5'-phosphosulfate reductase)	EP1108790 WO0100842	AX123178 AX066001
cysK	Cysteine Synthase EC 4.2.99.8 (cysteine synthase)	EP1108790 WO0100843	AX122901 AX063963
cysN	Sulfate Adenylyltransferase sub-unit I EC 2.7.7.4 (sulfate adenylyltransferase)	EP1108790	AX123176 AX127152
cysQ	Transport protein CysQ (transporter cysQ)	EP1108790 WO0100805	AX127145 AX066423
dps	DNA Protection Protein (protection during starvation protein)	EP1108790	AX127153
eno	Enolase EC 4.2.1.11 (enolase)	EP1108790 WO0100844 EP1090998 Hermann et al., Electrophoresis 19:3217-3221 (1998)	AX127146 AX064945 AX136862
fda	Fructose Bisphosphate Aldolase EC 4.1.2.13 (fructose bisphosphate aldolase)	van der Osten et al., Molecular Microbiology 3:1625-1637 (1989)	X17313
gap	Glyceraldehyde 3-Phosphate Dehydrogenase	EP1108790	AX127148

	EC 1.2.1.12 (glyceraldehyde 3-phosphate dehydrogenase)	WO0100844 Eikmanns et al., Journal of Bacteriology 174:6076-6086(1992)	AX064941 X59403
gap2	Glyceraldehyde 3-Phosphate Dehydrogenase EC 1.2.1.12 (glyceraldehyde 3-phosphate dehydrogenase 2)	EP1108790 WO0100844	AX127146 AX064939
gdh	Glutamate Dehydrogenase EC 1.4.1.4 (glutamate dehydrogenase)	EP1108790 WO0100844 Boermann et al., Molecular Microbiology 6:317-326 (1992) Guyonvarch et al, NCBI	AX127150 AX063811 X59404 X72855
gnd	6-Phosphogluconate Dehydrogenase EC 1.1.1.44 (6-phosphogluconate dehydrogenase)	EP1108790 WO0100844	AX127147 AX121689 AX065125
hom	Homoserine Dehydrogenase EC 1.1.1.3 (homoserine dehydrogenase)	Peoples et al., Molecular Microbiology 2:63-72 (1988)	Y00546
hom^{FBR}	Homoserine Dehydrogenase feedback resistant (fbr) EC 1.1.1.3 (homoserine dehydrogenase fbr)	Reinscheid et al., Journal of Bacteriology 173:3228-30 (1991)	
lysC	Aspartate Kinase EC 2.7.2.4 (aspartate kinase)	EP1108790 WO0100844 Kalinowski et al., Molecular Microbiology 5:1197-204 (1991)	AX120365 AX063743 X57226
$lysC^{FBR}$	Aspartate Kinase feedback resistent (fbr) EC 2.7.2.4 (aspartate kinase fbr)	see Table 2	
msiK	Sugar Importer (multiple sugar import protein)	EP1108790	AX120892
opcA	Glucose 6-Phosphate Dehydrogenase (subunit of glucose 6-phosphate dehydrogenase)	WO0104325	AX076272
oxyR	Transcription Regulator (transcriptional regulator)	EP1108790	AX122198 AX127149
ppc^{FBR}	Phosphoenol Pyruvate Carboxylase feedback resistent EC 4.1.1.31 (phosphoenol pyruvate carboxylase feedback resistant)	EP0723011 WO0100852	
ppc	Phosphoenol Pyruvate Carboxylase EC 4.1.1.31 (phosphoenol pyruvate carboxylase)	EP1108790 O'Reagan et al., Gene	AX127148 AX123554 M25819

		77(2):237-251(1989)	
pgk	Phosphoglycerate Kinase EC 2.7.2.3 (phosphoglycerate kinase)	EP1108790 WO0100844 Eikmanns, Journal of Bacteriology 174:6076-6086 (1992)	AX121838 AX127148 AX064943 X59403
pknA	Protein Kinase A (protein kinase A)	EP1108790	AX120131 AX120085
pknB	Protein Kinase B (protein kinase B)	EP1108790	AX120130 AX120085
pknD	Protein Kinase D (protein kinase D)	EP1108790	AX127150 AX122469 AX122468
pknG	Protein Kinase G (protein kinase G)	EP1108790	AX127152 AX123109
ppsA	Phosphoenol Pyruvate Synthase EC 2.7.9.2 (phosphoenol pyruvate synthase)	EP1108790	AX127144 AX120700 AX122469
ptsH	Phosphotransferase System Protein H EC 2.7.1.69 (phosphotransferase system component H)	EP1108790 WO0100844	AX122210 AX127149 AX069154
ptsI	Phosphotransferase System Enzyme I EC 2.7.3.9 (phosphotransferase system enzyme I)	EP1108790	AX122206 AX127149
ptsM	Glucose-specific Phosphotransferase System Enzyme II EC 2.7.1.69 (glucose phosphotransferase-system enzyme II)	Lee et al., FEMS Microbiology Letters 119(1-2):137-145 (1994)	L18874
pyc	Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase)	WO9918228 Peters-Wendisch et al., Microbiology 144:915-927 (1998)	A97276 Y09548
pyc P458S	Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) amino acid exchange P458S	EP1108790	
sigC	Sigma Factor C EC 2.7.7.6 (extracytoplasmic function alternative sigma factor C)	EP1108790	AX120368 AX120085
sigD	RNA Polymerase Sigma Factor D EC 2.7.7.6 (RNA polymerase sigma factor)	EP1108790	AX120753 AX127144
sigE	Sigma Factor E EC 2.7.7.6 (extracytoplasmic function alternative sigma factor E)	EP1108790	AX127146 AX121325
sigH	Sigma Factor H EC 2.7.7.6 (sigma factor SigH)	EP1108790	AX127145 AX120939

sigM	Sigma Factor M EC 2.7.7.6 (sigma factor SigM)	EP1108790	AX123500 AX127153
tal	Transaldolase EC 2.2.1.2 (transaldolase)	WO0104325	AX076272
thrB	Homoserine Kinase EC 2.7.1.39 (homoserine kinase)	Peoples et al., Molecular Microbiology 2:63-72 (1988)	Y00546
thrC	Threonine Synthase EC 4.2.99.2 (threonine synthase)	Han et al., Molecular Microbiology 4:1693-1702 (1990)	X56037
thrE	Threonine Exporter (threonine export carrier)	EP1085091	AX137526
thyA	Thymidylate Synthase EC 2.1.1.45 (thymidylate synthase)	EP1108790	AX121026 AX127145
tkt	Transketolase EC 2.2.1.1 (transketolase)	Ikeda et al., NCBI	AB023377
tpi	Triose Phosphate Isomerase EC 5.3.1.1 (triose phosphate isomerase)	Eikmanns, Journal of Bacteriology 174:6076-6086 (1992)	X59403
zwa1	Cell Growth Factor 1 (growth factor 1)	EP1111062	AX133781
zwf	Glucose 6-Phosphate 1-Dehydrogenase EC 1.1.1.49 (glucose 6-phosphate 1-dehydrogenase)	EP1108790 WO0104325	
zwf A213T	Glucose 6-Phosphate 1-Dehydrogenase EC 1.1.1.49 (glucose 6-phosphate 1-dehydrogenase) amino acid exchange A213T	EP1108790	AX127148 AX121827 AX076272

Table 7

Further gene sites for integration of open reading frames, genes and alleles of threonine production

Gene name	Description of the coded enzyme or protein	Reference	Access Number
ccpA1	Catabolite Control Protein (catabolite control protein A1)	WO0100844 EP1108790	AX065267 AX127147
ccpA2	Catabolite Control Protein (catabolite control protein A2)	WO0100844 EP1108790	AX065267 AX121594
citA	Sensor Kinase CitA (sensor kinase CitA)	EP1108790	AX120161
citB	Transcription Regulator CitB (transcription regulator CitB)	EP1108790	AX120163
citE	Citrate Lyase EC 4.1.3.6 (citrate lyase)	WO0100844 EP1108790	AX065421 AX127146
ddh	Diaminopimelate Dehydrogenase EC 1.4.1.16 (diaminopimelate dehydrogenase)	Ishino et al., Nucleic Acids Research 15: 3917 (1987) EP1108790	S07384 AX127152
gluA	Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding protein)	Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)	X81191
gluB	Glutamate-binding Protein (glutamate-binding protein)	Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)	X81191
gluC	Glutamate Transport Permease (glutamate transport system permease)	Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)	X81191
gluD	Glutamate Transport Permease (glutamate transport system permease)	Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)	X81191
glyA	Glycine Hydroxymethyltransferase EC 2.1.2.1 (glycine hydroxymethyltransferase)	WO0100843	AX063861 AF327063
ilvA	Threonine Dehydratase EC 4.2.1.16 (threonine dehydratase)	Möckel et al., Journal of Bacteriology 174 (24), 8065-8072 (1992) EP1108790	A47044 L01508 AX127150
ilvBN	Acetolactate Synthase EC 4.1.3.18	Keilhauer et al., Journal of Bacteriology	A48648 L09232

	(acetolactate synthase)	175(17):5595-603 (1993) EP1108790	AX127147
ilvC	Reductoisomerase EC 1.1.1.86 (ketol-acid reductoisomerase)	Keilhauer et al., Journal of Bacteriology 175(17):5595-603 (1993) EP1108790	C48648 AX127147
ilvD	Dihydroxy-acid Dehydratase EC 4.2.1.9 (dihydroxy-acid dehydratase)	EP1006189	AX136925
luxR	Transcription Regulator LuxR (transcription regulator LuxR)	WO0100842 EP1108790	AX065953 AX123320
luxS	Histidine Kinase LuxS (histidine kinase LuxS)	EP1108790	AX123323 AX127153
lysR1	Transcription Regulator LysR1 (transcription regulator LysR1)	EP1108790	AX064673 AX127144
lysR2	Transcription Activator LysR2 (transcription regulator LysR2)	EP1108790	AX123312
lysR3	Transcription Regulator LysR3 (transcription regulator LysR3)	WO0100842 EP1108790	AX065957 AX127150
mdh	Malate Dehydrogenase EC 1.1.1.37 (malate dehydrogenase)	WO0100844	AX064895
menE	O-Succinylbenzoic Acid CoA Ligase EC 6.2.1.26 (O-succinylbenzoate CoA ligase)	WO0100843 EP1108790	AX064599 AX064193 AX127144
metA	Homoserine O-Acetyltransferase EC 2.3.1.31 (homoserine O-acetyltransferase)	Park et al., Molecular Cells 30;8(3):286-94 (1998) WO0100843 EP1108790	AX063895 AX127145
metD	Transcription Regulator MetD (transcription regulator MetD)	EP1108790	AX123327 AX127153
pck	Phosphoenol Pyruvate Carboxykinase (phosphoenol pyruvate carboxykinase)	WO0100844	AJ269506 AX065053
poxB	Pyruvate Oxidase EC 1.2.3.3 (pyruvate oxidase)	WO0100844 EP1096013	AX064959 AX137665
sigB	RNA Polymerase Transcription Factor (RNA polymerase transcription factor)	EP1108790	AX127149
zwa2	Cell Growth Factor 2	EP1106693	AX113822

	(growth factor 2)	EP1108790	AX127146

The invention accordingly also provides a process for the production of coryneform bacteria which produce L-methionine and/or L-threonine, characterized in that

a) the nucleotide sequence of a desired ORF, gene or allele of methionine production or threonine production, optionally including the expression and/or regulation signals, is isolated

b) at least two copies of the nucleotide sequence of the ORF, gene or allele of methionine production or threonine production are arranged in a row, preferably in tandem arrangement

c) the nucleotide sequence obtained according to b) is incorporated in a vector which does not replicate or replicates to only a limited extent in coryneform bacteria,

d) the nucleotide sequence according to b) or c) is transferred into coryneform bacteria, and

e) coryneform bacteria which have at least two copies of the desired ORF, gene or allele of methionine or threonine production at the particular desired natural site instead of the singular copy of the ORF, gene or allele originally present are isolated, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remaining at the particular natural site (locus), and optionally

f) at least a third copy of the open reading frame (ORF), gene or allele of methionine production or threonine

production in question is introduced at a further gene site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remaining at the further gene site.

The invention also provides coryneform bacteria, in particular of the genus Corynebacterium, which produce L-valine, characterized in that

a) instead of the singular copy of an open reading frame (ORF), a gene or allele of valine production naturally present at the particular desired site (locus), these have at least two copies of the said open reading frame (ORF), gene or allele, preferably in tandem arrangement, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the particular site, and in that these

b) optionally have at least a third copy of the open reading frame (ORF), gene or allele of valine production mentioned at a further gene site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the further gene site.

The invention also furthermore provides a process for the preparation of L-valine, which comprises the following steps:

a) fermentation of coryneform bacteria, in particular of the genus Corynebacterium, which

i) instead of the singular copy of an open reading frame (ORF), gene or allele of valine production present at the particular desired site (locus), have at least two copies of the open reading frame (ORF), gene or allele in question, preferably in tandem arrangement, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the particular site, and

ii) optionally have at least a third copy of the open reading frame (ORF), gene or allele of valine production in question at a further gene site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the further gene site,

under conditions which allow expression of the said open reading frames (ORFs), genes or alleles,

b) concentration of the L-valine in the fermentation broth,

c) isolation of the L-valine from the fermentation broth, optionally

d) with constituents from the fermentation broth and/or the biomass to the extent of > (greater than) 0 to 100%.

A "copy of an open reading frame (ORF), gene or allele of valine production" is to be understood as meaning all the, preferably endogenous, open reading frames, genes or alleles of which enhancement/over-expression can have the effect of improving valine production.

These include, inter alia, the following open reading frames, genes or alleles: brnE, brnF, brnEF, cstA, cysD, dps, eno, fda, gap, gap2, gdh, ilvB, ilvN, ilvBN, ilvC, ilvD, ilvE msiK, pgk, ptsH, ptsI, ptsM, sigC, sigD, sigE, sigH, sigM, tpi and zwa1. These are summarized and explained in Table 8. These include in particular the acetolactate synthase ilvBN alleles which code for a valine-resistant.

The at least third, optionally fourth or fifth copy of the open reading frame (ORF), gene or allele of valine production in question can be integrated at a further site. The following open reading frames, genes or nucleotide sequences, inter alia, can be used for this: aecD, ccpA1, ccpA2, citA, citB, citE, ddh, gluA, gluB, gluC, gluD, glyA, ilvA, luxR, lysR1, lysR2, lysR3, panB, panC, poxB and zwa2. These are summarized and explained in Table 9. Intergenic regions in the chromosome, that is to say nucleotide sequences without a coding function, can furthermore be used. Finally, prophages or defective phages contained in the chromosome can be used for this.

Table 8

Open reading frames, genes and alleles of valine production

Name	Description of the coded enzyme or protein	Reference	Access Number
brnEF	Export of branched-chain amino acids (branched chain amino acid export)	EP1096010 Kennerknecht et al., NCBI	 AF454053
cstA	Carbon Starvation Protein A (carbon starvation protein A)	EP1108790 WO0100804	AX120811 AX066109
dps	DNA Protection Protein (protection during starvation protein)	EP1108790	AX127153
eno	Enolase EC 4.2.1.11 (enolase)	EP1108790 WO0100844 EP1090998 Hermann et al., Electrophoresis 19:3217-3221 (1998)	AX127146 AX064945 AX136862
fda	Fructose Bisphosphate Aldolase EC 4.1.2.13 (fructose bisphosphate aldolase)	van der Osten et al., Molecular Microbiology 3:1625-1637 (1989)	X17313
gap	Glyceraldehyde 3-Phosphate Dehydrogenase EC 1.2.1.12 (glyceraldehyde 3-phosphate dehydrogenase)	EP1108790 WO0100844 Eikmanns et al., Journal of Bacteriology 174:6076-6086(1992)	AX127148 AX064941 X59403
gap2	Glyceraldehyde 3-Phosphate Dehydrogenase EC 1.2.1.12 (glyceraldehyde 3-phosphate dehydrogenase 2)	EP1108790 WO0100844	AX127146 AX064939
gdh	Glutamate Dehydrogenase EC 1.4.1.4 (glutamate dehydrogenase)	EP1108790 WO0100844 Boermann et al., Molecular Microbiology 6:317-326 (1992); Guyonvarch et al., NCBI	AX127150 AX063811 X59404 X72855
ilvBN	Acetolactate Synthase EC 4.1.3.18 (acetolactate synthase)	Keilhauer et al., Journal of Bacteriology 175(17):5595-603 (1993) EP1108790	L09232 AX127147
ilvC	Isomeroreductase EC 1.1.1.86	Keilhauer et al., Journal of	C48648 AX127147

	(acetohydroxy acid isomeroreductase)	Bacteriology 175(17):5595-603 (1993) EP1108790	
ilvD	Dihydroxy-acid Dehydratase EC 4.2.1.9 (dihydroxy acid dehydratase)	EP1006189	AX136925
ilvE	Transaminase B EC 2.6.1.42 (transaminase B)	EP1108790	AX127150 AX122498
msiK	Sugar Importer (multiple sugar import protein)	EP1108790	AX120892
pgk	Phosphoglycerate Kinase EC 2.7.2.3 (phosphoglycerate kinase)	EP1108790 WO0100844 Eikmanns, Journal of Bacteriology 174:6076-6086 (1992)	AX121838 AX127148 AX064943 X59403
ptsH	Phosphotransferase System Protein H EC 2.7.1.69 (phosphotransferase system component H)	EP1108790 WO0100844	AX122210 AX127149 AX069154
ptsI	Phosphotransferase System Enzyme I EC 2.7.3.9 (phosphotransferase system enzyme I)	EP1108790	AX122206 AX127149
ptsM	Glucose-specific Phosphotransferase System Enzyme II EC 2.7.1.69 (glucose phosphotransferase-system enzyme II)	Lee et al., FEMS Microbiology Letters 119(1-2):137-145 (1994)	L18874
sigC	Sigma Factor C EC 2.7.7.6 (extracytoplasmic function alternative sigma factor C)	EP1108790	AX120368 AX120085
sigD	RNA Polymerase Sigma Factor D EC 2.7.7.6 (RNA polymerase sigma factor)	EP1108790	AX120753 AX127144
sigE	Sigma Factor E EC 2.7.7.6 (extracytoplasmic function alternative sigma factor E)	EP1108790	AX127146 AX121325
sigH	Sigma Factor H EC 2.7.7.6 (sigma factor SigH)	EP1108790	AX127145 AX120939
sigM	Sigma Factor M EC 2.7.7.6 (sigma factor SigM)	EP1108790	AX123500 AX127153
tpi	Triose Phosphate Isomerase EC 5.3.1.1 (triose phosphate isomerase)	Eikmanns, Journal of Bacteriology 174:6076-6086 (1992)	X59403
zwa1	Cell Growth Factor 1 (growth factor 1)	EP1111062	AX133781

Table 9

Further gene sites for integration of open reading frames, genes and alleles of valine production

Gene name	Description of the coded enzyme or protein	Reference	Access Number
aecD	beta C-S Lyase EC 2.6.1.1 (beta C-S lyase)	Rossol et al., Journal of Bacteriology 174(9):2968-77 (1992)	M89931
ccpA1	Catabolite Control Protein (catabolite control protein A1)	WO0100844 EP1108790	AX065267 AX127147
ccpA2	Catabolite Control Protein (catabolite control protein A2)	WO0100844 EP1108790	AX065267 AX121594
citA	Sensor Kinase CitA (sensor kinase CitA)	EP1108790	AX120161
citB	Transcription Regulator CitB (transcription regulator CitB)	EP1108790	AX120163
citE	Citrate Lyase EC 4.1.3.6 (citrate lyase)	WO0100844 EP1108790	AX065421 AX127146
ddh	Diaminopimelate Dehydrogenase EC 1.4.1.16 (diaminopimelate dehydrogenase)	Ishino et al., Nucleic Acids Research 15: 3917 (1987) EP1108790	S07384 AX127152
gluA	Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding protein)	Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)	X81191
gluB	Glutamate-binding Protein (glutamate-binding protein)	Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)	X81191
gluC	Glutamate Transport Permease (glutamate transport system permease)	Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)	X81191
gluD	Glutamate Transport Permease (glutamate transport system permease)	Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)	X81191
glyA	Glycine Hydroxymethyltransferase EC 2.1.2.1 (glycine hydroxymethyltransferase)	WO0100843	AX063861 AF327063
ilvA	Threonine Dehydratase EC 4.2.1.16 (threonine dehydratase)	Möckel et al., Journal of Bacteriology 174 (24), 8065-8072 (1992)	A47044 L01508 AX127150

		EP1108790	
luxR	Transcription Regulator LuxR (transcription regulator LuxR)	WO0100842 EP1108790	AX065953 AX123320
lysR1	Transcription Regulator LysR1 (transcription regulator LysR1)	EP1108790	AX064673 AX127144
lysR2	Transcription Activator LysR2 (transcription regulator LysR2)	EP1108790	AX123312
lysR3	Transcription Regulator LysR3 (transcription regulator LysR3)	WO0100842 EP1108790	AX065957 AX127150
panB	Ketopantoate Hydroxymethyltransferase EC 2. 1. 2. 11 (ketopantoate hydroxymethyltransferase)	US6177264	X96580
panC	Pantothenate Synthetase EC 6.3.2.1 (pantothenate synthetase)	US6177264	X96580
poxB	Pyruvate Oxidase EC 1.2.3.3 (pyruvate oxidase)	WO0100844 EP1096013	AX064959 AX137665
zwa2	Cell Growth Factor 2 (growth factor 2)	EP1106693 EP1108790	AX113822 AX127146

The invention accordingly also provides a process for the production of coryneform bacteria which produce L-valine, characterized in that

a) the nucleotide sequence of a desired ORF, gene or allele of valine production, optionally including the expression and/or regulation signals, is isolated

b) at least two copies of the nucleotide sequence of the ORF, gene or allele of valine production are arranged in a row, preferably in tandem arrangement

c) the nucleotide sequence obtained according to b) is incorporated in a vector which does not replicate or replicates to only a limited extent in coryneform bacteria,

d) the nucleotide sequence according to b) or c) is transferred into coryneform bacteria, and

e) coryneform bacteria which have at least two copies of the desired open ORF, gene or allele of valine production at the particular desired natural site instead of the singular copy of the ORF, gene or allele originally present are isolated, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remaining at the particular natural site (locus), and optionally

f) at least a third copy of the open reading frame (ORF), gene or allele of valine production in question is introduced at a further gene site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remaining at the further gene site.

During work on the present invention, it was possible to incorporate two copies, arranged in tandem, of an $lysC^{FBR}$ allele at the lysC gene site of Corynebacterium glutamicum such that no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remain at the lysC gene site. Such a strain is, for example, the strain DSM13992$lysC^{FBR}$::$lysC^{FBR}$.

The plasmid pK18mobsacB2xlysCSma2/1, with the aid of which two copies of an $lysC^{FBR}$ allele can be incorporated into the lysC gene site of Corynebacterium glutamicum, is shown in Figure 1.

During work on the present invention, it was furthermore possible to incorporate two copies, arranged in tandem, of the lysE gene at the lysE gene site of Corynebacterium glutamicum such that no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remained at the lysE gene site. Such a strain is, for example, the strain ATCC21513_17lysE::lysE.

A plasmid with the aid of which two copies of an lysE gene can be incorporated into the lysE gene site of Corynebacterium glutamicum is shown in Figure 2. It carries the name pK18mobsacB2xlysESma1/1.

During work on the present invention, finally, it was possible to incorporate two copies, arranged in tandem, of the zwa1 gene at the zwa1 gene site of Corynebacterium glutamicum such that no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remained at the zwa1 gene site. Such a strain is, for example, the strain ATCC21513_17zwa1::zwa1.

A plasmid with the aid of which two copies of a zwa1 gene can be incorporated into the zwa1 gene site of Corynebacterium glutamicum is shown in Figure 3. It carries the name pK18mobsacBzwa1zwa1.

The coryneform bacteria produced according to the invention can be cultured continuously or discontinuously in the batch process (batch culture) or in the fed batch (feed process) or repeated fed batch process (repetitive feed process) for the purpose of production of chemical compounds. A summary of known culture methods is described

in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, such as e.g. soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as e.g. palmitic acid, stearic acid and linoleic acid, alcohols, such as e.g. glycerol and ethanol, and organic acids, such as e.g. acetic acid or lactic acid, can be used as the source of carbon. These substances can be used individually or as a mixture.

Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as e.g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be

employed in addition to the above-mentioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH of the culture. Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, such as e.g. antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as e.g. air, are introduced into the culture. The temperature of the culture is usually 20ºC to 45ºC, and preferably 25ºC to 40ºC. Culturing is continued until a maximum of the desired chemical compound has formed. This target is usually reached within 10 hours to 160 hours.

It has been found that the coryneform bacteria according to the invention, in particular the coryneform bacteria which produce L-lysine, have an unexpectedly high stability. They were stable for at least 10-20, 20-30, 30-40, 40-50, preferably at least 50-60, 60-70, 70-80 and 80-90 generations or cell division cycles.

The following microorganisms have been deposited:

The Corynebacterium glutamicum strain DSM13992lysC^{FBR}::lysC^{FBR} was deposited in the form of a pure culture on 5th June 2002 under number DSM15036 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty.

The plasmid pK18mobsacB2xlysCSma2/1 was deposited in the form of a pure culture of the strain E. coli DH5αmcr/ pK18mobsacB2xlysCSma2/1 (= DH5alphamcr/ pK18mobsacB2xlysCSma2/1) on 20th April 2001 under number DSM14244 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty.

The Corynebacterium glutamicum strain ATCC21513_17lysE::lysE was deposited in the form of a pure culture on 5th June 2002 under number DSM15037 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty.

The Corynebacterium glutamicum strain ATCC21513_17zwa1::zwa1 was deposited in the form of a pure culture on 5th June 2002 under number DSM15038 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty.

Example 1

Generation of a tandem duplication of the $lysC^{FBR}$ allele lysC T311I in the chromosome of Corynebacterium glutamicum

1.1. Construction of the tandem vector pK18mobsacB2xlysCSma2/1

From the Corynebacterium glutamicum strain DSM13994, chromosomal DNA is isolated by the conventional methods (Eikmanns et al., Microbiology 140: 1817 - 1828 (1994)).

The strain DSM13994 was produced by multiple, non-directed mutagenesis, selection and mutant selection from C. glutamicum ATCC13032. The strain is resistant to the lysine analogue S-(2-aminoethyl)-L-cysteine and has a feed back-resistant aspartate kinase which is insensitive to

inhibition by a mixture of lysine and threonine (in each case 25 mM). The nucleotide sequence of the $lysC^{FBR}$ allele is shown as SEQ ID NO:3. It is also called lysC T311I in the following. The amino acid sequence of the aspartate kinase protein coded is shown as SEQ ID NO:4. A pure culture of this strain was deposited on 16th January 2001 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty.

With the aid of the polymerase chain reaction, a DNA section which carries the lysC gene or allele is amplified. On the basis of the sequence of the lysC gene known for C. glutamicum (Kalinowski et al., Molecular Microbiology, 5 (5), 1197 - 1204(1991); Accession Number X57226), the following primer oligonucleotides were chosen for the PCR:

lysC1beg (SEQ ID No: 15):
5` TA(G GAT CC)T CCG GTG TCT GAC CAC GGT G 3`

lysC2end: (SEQ ID NO: 16):
5` AC(G GAT CC)G CTG GGA AAT TGC GCT CTT CC 3`

The primers shown are synthesized by MWG Biotech and the PCR reaction is carried out by the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press). The primers allow amplification of a DNA section of approx. 1.7 kb in length, which carries the lysC gene or allele. The primers moreover contain the sequence for a cleavage site of the restriction endonuclease BamHI, which is marked by parentheses in the nucleotide sequence shown above.

The amplified DNA fragment of approx. 1.7 kb in length which carries the $lysC^{FBR}$ allele lysC T311I of the strain DSM13994 is identified by electrophoresis in a 0.8% agarose gel, isolated from the gel and purified by conventional methods (QIAquick Gel Extraction Kit, Qiagen, Hilden).

Ligation of the fragment is then carried out by means of the Topo TA Cloning Kit (Invitrogen, Leek, The Netherlands, Cat. Number K4600-01) in the vector pCRII-TOPO. The ligation batch is transformed in the E. coli strain TOP10 (Invitrogen, Leek, The Netherlands). Selection of plasmid-carrying cells is made by plating out the transformation batch on kanamycin (50 mg/l)-containing LB agar with X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside, 64 mg/l).

The plasmid obtained is checked by means of restriction cleavage, after isolation of the DNA, and identified in agarose gel. The resulting plasmid is called pCRIITOPOlysC.

The nucleotide sequence of the amplified DNA fragment or PCR product is determined by the dideoxy chain termination method of Sanger et al. (Proceedings of the National Academy of Sciences USA, 74:5463-5467 (1977)) using the "ABI Prism 377" sequencing apparatus of PE Applied Biosystems (Weiterstadt, Germany). The sequence of the coding region of the PCR product is shown in SEQ ID No:3. The amino acid sequence of the associated aspartate kinase protein is shown in SEQ ID NO:4.

The base thymine is found at position 932 of the nucleotide sequence of the coding region of the $lysC^{FBR}$ allele of strain DSM13994 (SEQ ID NO:3). The base cytosine is found at the corresponding position of the wild-type gene (SEQ ID NO:1).

The amino acid isoleucine is found at position 311 of the amino acid sequence of the aspartate kinase protein of strain DSM13994 (SEQ ID No:4). The amino acid threonine is found at the corresponding position of the wild-type protein (SEQ ID No:2).

The lysC allele, which contains the base thymine at position 932 of the coding region and accordingly codes for

an aspartate kinase protein which contains the amino acid isoleucine at position 311 of the amino acid sequence, is called the $lysC^{FBR}$ allele lysC T311I in the following.

The plasmid pCRIITOPOlysC, which carries the $lysC^{FBR}$ allele lysC T311I, was deposited in the form of a pure culture of the strain E. coli TOP 10/pCRIITOPOlysC under number DSM14242 on 20th April 2001 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty.

Plasmid DNA was isolated from the strain DSM14242, which carries the plasmid pCRIITOPOlysC, and cleaved with the restriction enzyme BamHI (Amersham-Pharmacia, Freiburg, Germany), after separation in an agarose gel (0.8%) the $lysC^{FBR}$-containing DNA fragment approx. 1.7 kb long is isolated from the agarose gel with the aid of the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), and the overhanging ends are completed with Klenow polymerase (Boehringer Mannheim) and employed for ligation with the mobilizable cloning vector pK18mobsacB described by Schäfer et al., Gene, 14, 69-73 (1994). This is cleaved beforehand with the restriction enzyme SmaI and dephosphorylated with alkaline phosphatase (Alkaline Phosphatase, Boehringer Mannheim), mixed with the $lysC^{FBR}$-containing fragment of approx. 1.7 kb and the mixture is treated with T4 DNA Ligase (Amersham-Pharmacia, Freiburg, Germany).

The E. coli strain DH5α (Grant et al.; Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649) is then transformed with the ligation batch (Hanahan, In. DNA Cloning. A Practical Approach. Vol. 1, ILR-Press, Cold Spring Harbor, New York, 1989). Selection of plasmid-carrying cells is made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which was supplemented with 25 mg/l kanamycin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage with the enzyme HindIII and subsequent agarose gel electrophoresis. The plasmid is called pK18mobsacB1xlysCSma2.

In a second step, the plasmid pCRII-TOPOlysC is in turn cleaved with the restriction enzyme BamHI (Amersham-Pharmacia, Freiburg, Germany), after separation in an agarose gel (0.8%) the $lysC^{FBR}$-containing fragment of approx. 1.7 kb was isolated from the agarose gel with the aid of the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and employed for ligation with the vector pK18mobsacB1xlysCSma2 described in this Example. This is cleaved beforehand with the restriction enzyme BamHI and dephosphorylated with alkaline phosphatase (Alkaline Phosphatase, Boehringer Mannheim), mixed with the $lysC^{FBR}$-containing fragment of approx. 1.7 kb and the mixture is treated with T4 DNA Ligase (Amersham-Pharmacia, Freiburg, Germany).

The E. coli strain DH5α (Grant et al.; Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649) is then transformed with the ligation batch (Hanahan, In. DNA Cloning. A Practical Approach. Vol. 1, ILR-Press, Cold Spring Harbor, New York, 1989). Selection of plasmid-carrying cells is made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which was supplemented with 25 mg/l kanamycin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage with the enzyme HindIII and subsequent agarose gel electrophoresis. The plasmid is called pK18mobsacB2xlysCSma2/1. A map of the plasmid is shown in Figure 1.

The plasmid pK18mobsacB2xlysCSma2/1 was deposited in the form of a pure culture of the strain E. coli DH5αmcr/ pK18mobsacB2xlysCSma2/1 (= DH5alphamcr/ pK18mobsacB2xlysCSma2/1) on 20th April 2001 under number DSM14244 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty.

1.2. Generation of a tandem duplication of the $lysC^{FBR}$ allele lysC T311I in C. glutamicum strain DSM13992

The vector pK18mobsacB2xlysCSma2/1 mentioned in Example 1.1 is transferred by a modified protocol of Schäfer et al. (1990 Journal of Microbiology 172: 1663-1666) into the C. glutamicum strain DSM13992.

The Corynebacterium glutamicum strain DSM13992 was produced by multiple, non-directed mutagenesis, selection and mutant selection from C. glutamicum ATCC13032. The strain is resistant to the antibiotic streptomycin and phenotypically resistant to the lysine analogue S-(2-aminoethyl)-L-cysteine. However, the strain has a wild-type aspartate kinase (see SEQ ID NO:1 and 2), which is sensitive to inhibition by a mixture of lysine and threonine (in each case 25 mM). A pure culture of this strain was deposited on 16th January 2001 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty.

The vector pK18mobsacB2xlysCSma2/1 cannot replicate independently in DSM13992 and is retained in the cell only if it has integrated into the chromosome.

Selection of clones with integrated pK18mobsacB2xlysCSma2/1 is carried out by plating out the conjugation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which was supplemented with 15 mg/l kanamycin and 50 mg/l

nalidixic acid. Clones which have grown on are plated out on LB agar plates with 25 mg/l kanamycin and incubated for 16 hours at 33°C. To achieve excision of the plasmid with only one copy of the lysC gene, the clones are cultured on LB agar with 10% sucrose, after incubation for 16 hours in LB liquid medium. The plasmid pK18mobsacB contains a copy of the sacB gene, which converts sucrose into levan sucrase, which is toxic to C. glutamicum.

Only those clones in which the pK18mobsacB2xlysCSma2/1 integrated has been excised again therefore grow on LB agar with sucrose. Approximately 40 to 50 colonies are tested for the phenotype "growth in the presence of sucrose" and "non-growth in the presence of kanamycin". During the excision, either two copies of the lysC gene or only one can be excised together with the plasmid.

To demonstrate that two copies of lysC have remained in the chromosome, approximately 20 colonies which show the phenotype "growth in the presence of sucrose" and "non-growth in the presence of kanamycin" are investigated with the aid of the polymerase chain reaction by the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press). A DNA fragment which carries the lysC gene and surrounding regions is amplified here from the chromosomal DNA of the colonies. The following primer oligonucleotides are chosen for the PCR.

lysCK1 (SEQ ID NO: 5):
5` TCG GTG TCA TCA GAG CAT TG 3`

lysCK2 (SEQ ID NO: 6):
5` TCG GTT GCC TGA GTA ATG TC 3`

The primers allow amplification of a DNA fragment approx. 1.9 kb in size in control clones with the original lysC locus. In clones with a second copy of the lysC gene in the

chromosome at the lysC locus, DNA fragments with a size of approx. 3.6 kb are amplified.

The amplified DNA fragments are identified by means of electrophoresis in a 0.8% agarose gel. On the basis of the amplified fragment length, a distinction was made between clones with one chromosomal lysC gene copy and clones with two chromosomal lysC gene copies.

10 clones with two complete copies of the lysC gene on the chromosome are investigated with the aid of the LightCycler of Roche Diagnostics (Mannheim, Germany) in order to demonstrate whether the two copies are $lysC^{FBR}$ alleles with the mutation lysC T311I or whether the original wild-type lysC is present alongside an $lysC^{FBR}$ allele lysC T311I. The LightCycler is a combined apparatus of Thermocycler and flourimeter.

A DNA section approx. 500 bp in length which contains the mutation site is amplified in the first phase by means of a PCR (Innis et al., PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press) using the following primer oligonucleotides.

LC-lysC1-fbr (SEQ ID No: 7):
5` aaccgttctgggtatttccg 3`

LC-lysC2-fbr (SEQ ID No: 8):
5` tccatgaactctgcggtaac 3`

In the second phase, with two additional oligonucleotides of different lengths and marked with different fluorescent dyestuffs (Lightcycler(LC)-Red640 and fluorescein), which hybridize in the region of the mutation site, the presence of the mutation is detected with the aid of the "Fluorescence Resonance Energy Transfer" method (FRET) using a melting curve analysis (Lay et al., Clinical Chemistry, 43:2262-2267 (1997)).

lysC311-C (SEQ ID No: 9):
5` LC-Red640 - gcaggtgaagatgatgtcggt - (P) 3`

lysC311-A (SEQ ID No: 10):
5` tcaagatctccatcgcgcggcggccgtcggaacga - fluorescein 3`

The primers shown are synthesized for the PCR by MWG Biotech and oligonucleotides shown for the hybridization are synthesized by TIB MOLBIOL (Berlin, Germany).

A clone which contains the base thymine at position 932 of the coding regions of the two lysC copies and thus has two $lysC^{FBR}$ alleles lysC T311I was identified in this manner.

The strain was called C. glutamicum DSM13992$lysC^{FBR}$::$lysC^{FBR}$.

The strain was deposited as C. glutamicum DSM13992$lysC^{FBR}$::$lysC^{FBR}$ on 5th June 2002 under number DSM15036 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty.

Example 2

Generation of a tandem duplication of the lysE gene in the chromosome of Corynebacterium glutamicum

2.1. Construction of the tandem vector pK18mobsacB2xlysESma1/1

Plasmid DNA was isolated from the Escherichia coli strain DSM12871 (EP-A-1067193), which carries the plasmid pEC7lysE.

The plasmid contains the lysE gene which codes for lysine export. A pure culture of this strain was deposited on 10th June 1999 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty.

The plasmid pEC71lysE is cleaved with the restriction enzyme BamHI (Amersham-Pharmacia, Freiburg, Germany), after separation in an agarose gel (0.8%) the lysE fragment of approx. 1.1 kb is isolated from the agarose gel with the aid of the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), and the overhanging ends are completed with Klenow polymerase (Boehringer Mannheim) and employed for ligation with the mobilizable cloning vector pK18mobsacB described by Schäfer et al., Gene, 14, 69-73 (1994). This is cleaved beforehand with the restriction enzyme SmaI and dephosphorylated with alkaline phosphatase (Alkaline Phosphatase, Boehringer Mannheim), mixed with the lysE fragment of approx. 1.1 kb and the mixture is treated with T4 DNA Ligase (Amersham-Pharmacia, Freiburg, Germany).

The E. coli strain DH5α (Grant et al.; Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649) is then transformed with the ligation batch (Hanahan, In. DNA Cloning. A Practical Approach. Vol. 1, ILR-Press, Cold Spring Harbor, New York, 1989). Selection of plasmid-carrying cells is made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which was supplemented with 25 mg/l kanamycin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage with the enzymes BamHI and EcoRI and subsequent agarose gel electrophoresis. The plasmid is called pK18mobsacB1xlysESma1.

In a second step, the plasmid pEC7lysE is in turn cleaved with the restriction enzyme BamHI (Amersham-Pharmacia, Freiburg, Germany), after separation in an agarose gel (0.8%) the lysE fragment of approx. 1.1 kb was isolated from the agarose gel with the aid of the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and employed for ligation with the vector pK18mobsacB1xlysESma1 described in

this Example. This is cleaved beforehand with the restriction enzyme BamHI and dephosphorylated with alkaline phosphatase (Alkaline Phosphatase, Boehringer Mannheim), mixed with the lysE fragment of approx. 1.1 kb and the mixture is treated with T4 DNA Ligase (Amersham-Pharmacia, Freiburg, Germany).

The E. coli strain DH5α (Grant et al.; Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649) is then transformed with the ligation batch (Hanahan, In. DNA Cloning. A Practical Approach. Vol. 1, ILR-Press, Cold Spring Harbor, New York, 1989). Selection of plasmid-carrying cells is made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which was supplemented with 25 mg/l kanamycin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage with the enzymes EcoRI and SalI or ScaI and subsequent agarose gel electrophoresis. The plasmid is called pK18mobsacB2xlysESma1/1. A map of the plasmid is shown in Figure 2.

2.2. Generation of a tandem duplication of the lysE gene in C. glutamicum strain ATCC21513_17

The vector pK18mobsacB2xlysESma1/1 mentioned in Example 2.1 is transferred by a modified protocol of Schäfer et al. (1990 Journal of Microbiology 172: 1663-1666) into the C. glutamicum strain ATCC21513_17.

The Corynebacterium glutamicum strain ATCC21513_17 was produced by multiple, non-directed mutagenesis, selection and mutant selection from C. glutamicum ATCC21513. The strain is resistant to the lysine analogue S-(2-aminoethyl)-L-cysteine and both leucine- and homoserine-prototrophic.

The vector cannot replicate independently in ATCC21513_17 and is retained in the cell only if it has integrated into the chromosome.

Selection of clones with integrated pK18mobsacB2xlysESma1/1 is carried out by plating out the conjugation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which was supplemented with 15 mg/l kanamycin and 50 mg/l nalidixic acid. Clones which have grown on are plated out on LB agar plates with 25 mg/l kanamycin and incubated for 16 hours at 33°C. To achieve excision of the plasmid with only one copy of the lysE gene, the clones are cultured on LB agar with 10% sucrose, after incubation for 16 hours in LB liquid medium. The plasmid pK18mobsacB contains a copy of the sacB gene, which converts sucrose into levan sucrase, which is toxic to C. glutamicum.

Only those clones in which the pK18mobsacB2xlysESma1/1 integrated has been excised again therefore grow on LB agar with sucrose. Approximately 40 to 50 colonies are tested for the phenotype "growth in the presence of sucrose" and "non-growth in the presence of kanamycin". During the excision, either two copies of the lysE gene or only one can be excised together with the plasmid.

To demonstrate that two copies of lysE have remained in the chromosome, approximately 20 colonies which show the phenotype "growth in the presence of sucrose" and "non-growth in the presence of kanamycin" are investigated with the aid of the polymerase chain reaction by the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press). A DNA fragment which carries the lysE gene and surrounding regions is amplified here from the chromosomal DNA of the colonies. The following primer oligonucleotides are chosen for the PCR.

lysEK-1 (SEQ ID NO: 11):
5` TGC TTG CAC AAG GAC TTC AC 3`

lysEK-2 (SEQ ID NO: 12):
5` TAT GGT CCG CAA GCT CAA TG 3`

The primers allow amplification of a DNA fragment approx. 1.2 kb in size in control clones with the original lysE locus. In clones with a second copy of the lysC gene in the chromosome at the lysE locus, DNA fragments with a size of approx. 2.3 kb are amplified.

The amplified DNA fragments are identified by means of electrophoresis in a 0.8% agarose gel. On the basis of the amplified fragment length, a distinction was made between clones with one chromosomal lysE gene copy and clones with two chromosomal lysE gene copies. It could thus be demonstrated that the strain ATCC21513_17 carries two complete copies of the lysE gene on the chromosome.

The strain was called C. glutamicum ATCC21513_17lysE::lysE.

The strain was deposited as C. glutamicum ATCC21513_17lysE::lysE on 5th June 2002 under number DSM15037 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty.

Example 3

Generation of a tandem duplication of the zwa1 gene in the chromosome of Corynebacterium glutamicum

3.1. Construction of the tandem vector pK18mobsacBzwa1zwa1

Plasmid DNA was isolated from the Escherichia coli strain DSM13115 (EP-A-1111062), which carries the plasmid pCR2.1zwa1exp.

The plasmid contains the zwa1 gene which codes for cell growth factor 1. A pure culture of this strain was deposited on 19th October 1999 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty.

The plasmid pCR2.1zwa1exp is cleaved with the restriction enzyme EcoRI (Amersham-Pharmacia, Freiburg, Germany), and after separation in an agarose gel (0.8%) the zwa1 fragment of 1 kb is isolated from the agarose gel with the aid of the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and employed for ligation with the mobilizable cloning vector pK18mobsacB described by Schäfer et al., Gene, 14, 69-73 (1994). This is cleaved beforehand with the restriction enzyme EcoRI and dephosphorylated with alkaline phosphatase (Alkaline Phosphatase, Boehringer Mannheim), mixed with the zwa1 fragment of 1 kb and the mixture is treated with T4 DNA Ligase (Amersham-Pharmacia, Freiburg, Germany).

The E. coli strain DH5α (Grant et al.; Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649) is then transformed with the ligation batch (Hanahan, In. DNA Cloning. A Practical Approach. Vol. 1, ILR-Press, Cold Spring Harbor, New York, 1989). Selection of plasmid-carrying cells is made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which was supplemented with 25 mg/l kanamycin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage with the enzyme NheI and subsequent agarose gel electrophoresis. Checking of the plasmid showed that two zwa1 fragments were cloned simultaneously and in the desired orientation in the cloning vector pK18mobsac.

The plasmid is called pK18mobsacBzwa1zwa1. A map of the plasmid is shown in Figure 3.

3.2. Generation of a tandem duplication of the zwa1 gene in C. glutamicum strain ATCC21513_17

The vector pK18mobsacBzwa1zwa1 mentioned in Example 3.1 is transferred by a modified protocol of Schäfer et al. (1990 Journal of Microbiology 172: 1663-1666) into the C. glutamicum strain ATCC21513_17.

The Corynebacterium glutamicum strain ATCC21513_17 was produced by multiple, non-directed mutagenesis, selection and mutant selection from C. glutamicum ATCC21513. The strain is resistant to the lysine analogue S-(2-aminoethyl)-L-cysteine and both leucine- and homoserine-prototrophic.

The vector cannot replicate independently in ATCC21513_17 and is retained in the cell only if it has integrated into the chromosome.

Selection of clones with integrated pK18mobsacBzwa1zwa1 is carried out by plating out the conjugation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which was supplemented with 15 mg/l kanamycin and 50 mg/l nalidixic acid. Clones which have grown on are plated out on LB agar plates with 25 mg/l kanamycin and incubated for 16 hours at 33°C. To achieve excision of the plasmid with only one copy of the zwa1 gene, the clones are cultured on LB agar with 10% sucrose, after incubation for 16 hours in LB liquid medium. The plasmid pK18mobsacB contains a copy of the sacB gene, which converts sucrose into levan sucrase, which is toxic to C. glutamicum.

Only those clones in which the pK18mobsacBzwa1zwa1 integrated has been excised again therefore grow on LB agar with sucrose. Approximately 40 to 50 colonies are tested

for the phenotype "growth in the presence of sucrose" and "non-growth in the presence of kanamycin". During the excision, either two copies of the zwa1 gene or only one can be excised together with the plasmid.

To demonstrate that two copies of zwa1 have remained in the chromosome, approximately 20 colonies which show the phenotype "growth in the presence of sucrose" and "non-growth in the presence of kanamycin" are investigated with the aid of the polymerase chain reaction by the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press). A DNA fragment which carries the zwa1 gene and surrounding regions is amplified here from the chromosomal DNA of the colonies. The following primer oligonucleotides are chosen for the PCR.

zwa1-A2 (SEQ ID NO: 13):
5` CAC TTG TCC TCA CCA CTT TC 3`

zwa1-E1 (SEQ ID NO: 14):
5` TTC TAC TGG GCG TAC TTT CG 3`

The primers allow amplification of a DNA fragment approx. 1.3 kb in size in control clones with the original zwa1 locus. In clones with a second copy of the zwa1 gene in the chromosome at the zwa1 locus, DNA fragments with a size of approx. 2.3 kb are amplified.

The amplified DNA fragments are identified by means of electrophoresis in a 0.8% agarose gel. On the basis of the amplified fragment length, a distinction was made between clones with one chromosomal zwa1 gene copy and clones with two chromosomal zwa1 gene copies. It could thus be demonstrated that the strain ATCC21513_17 carries two complete copies of the zwa1 gene on the chromosome.

The strain was called C. glutamicum ATCC21513_17zwa1::zwa1. The strain was deposited as C. glutamicum

ATCC21513_17zwa1::zwa1 on 5th June 2002 under number DSM15038 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty.

Example 4

Preparation of Lysine

The C. glutamicum strains DSM13992lysC^{FBR}::lysC^{FBR}, ATCC21513_17lysE::lysE and ATCC21513_17zwa1::zwa1 obtained in Examples 1 to 3 are cultured in a nutrient medium suitable for the production of lysine and the lysine content in the culture supernatant was determined.

For this, the strains are first incubated on an agar plate for 24 hours at 33°C. Starting from this agar plate culture, a preculture is seeded (10 ml medium in a 100 ml conical flask). The medium MM is used as the medium for the preculture. The preculture is incubated for 24 hours at 33°C at 240 rpm on a shaking machine. A main culture is seeded from this preculture such that the initial OD (660 nm) of the main culture is 0.1 OD. The Medium MM is also used for the main culture.

Medium MM

CSL	5 g/l
MOPS	20 g/l
Glucose (autoclaved separately)	50 g/l
Salts:	
$(NH_4)_2SO_4$	25 g/l
KH_2PO_4	0.1 g/l
$MgSO_4 * 7 H_2O$	1.0 g/l

$CaCl_2 * 2 H_2O$	10 mg/l
$FeSO_4 * 7 H_2O$	10 mg/l
$MnSO_4 * H_2O$	5.0 mg/l
Biotin (sterile-filtered)	0.3 mg/l
Thiamine * HCl (sterile-filtered)	0.2 mg/l
$CaCO_3$	25 g/l

The CSL (corn steep liquor), MOPS (morpholinopropanesulfonic acid) and the salt solution are brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions, as well as the $CaCO_3$ autoclaved in the dry state, are then added.

Culturing is carried out in a 10 ml volume in a 100 ml conical flask with baffles. Culturing is carried out at 33°C and 80% atmospheric humidity.

After 48 hours, the OD is determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The amount of lysine formed is determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivation with ninhydrin detection.

The result of the experiment is shown in Table 10.

Table 10

Strain	OD (660 nm)	Lysine HCl g/l
DSM13992	12.8	18.9
DSM13992lysC^{FBR}::lysC^{FBR}	12.0	21.6
ATCC21513_17	10.4	14.0
ATCC21513_17lysE::lysE	10.0	14.3
ATCC21513_17zwa1::zwa1	9.9	14.6

Brief Description of the Figures:

The base pair numbers stated are approximate values obtained in the context of reproducibility of measurements.

Figure 1: Map of the plasmid pK18mobsacB2xlysCSma2/1.

The abbreviations and designations used have the following meaning:

KmR: Kanamycin resistance gene

HindIII: Cleavage site of the restriction enzyme HindIII

BamHI: Cleavage site of the restriction enzyme BamHI

lysC: lysC^{FBR} allele lysC T311I

sacB: sacB gene

RP4mob: mob region with the replication origin for the transfer (oriT)

oriV: Replication origin V

Figure 2: Map of the plasmid pK18mobsacB2xlysESma1/1.

The abbreviations and designations used have the following meaning:

KanR: Kanamycin resistance gene

SalI: Cleavage site of the restriction enzyme SalI

BamHI: Cleavage site of the restriction enzyme BamHI

EcoRI: Cleavage site of the restriction enzyme EcoRI

ScaI: Cleavage site of the restriction enzyme ScaI

lysE: lysE gene

sacB: sacB gene

RP4mob: mob region with the replication origin for the transfer (oriT)

oriV: Replication origin V

Figure 3: Map of the plasmid pK18mobsacBzwa1zwa1.

The abbreviations and designations used have the following meaning:

KanR: Kanamycin resistance gene

EcoRI: Cleavage site of the restriction enzyme EcoRI

NheI: Cleavage site of the restriction enzyme NheI

zwa1:	zwa1 gene
sacB:	sacB gene
RP4mob:	mob region with the replication origin for the transfer (oriT)
oriV:	Replication origin V

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Degussa AG

Kantstr. 2

33790 Halle (Westf.)

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: DSM13992lysCFBR::lysCFBR	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 15036

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I. above was accompanied by:

(x) a scientific description
(x) a proposed taxonomic designation

(Mark with a cross where applicable).

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2002-06-05 (Date of the original deposit)[1].

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit)
and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): V. Weiss Date: 2002-06-06

[1] Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

Form DSMZ-BP/4 (sole page) 12/2001

BUDAPEST T... ...NTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Degussa AG
Kantstr. 2
33790 Halle (Westf.)

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Degussa AG Kantstr. 2 Address: 33790 Halle (Westf.)	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 15036 Date of the deposit or the transfer[1]: 2002-06-05
III. VIABILITY STATEMENT	
The viability of the microorganism identified under II above was tested on 2002-06-05 [2]. On that date, the said microorganism was (x)[3] viable ( )[3] no longer viable	
IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): V. Weiss Date: 2002-06-06

[1] Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
[2] In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.
[3] Mark with a cross the applicable box.
[4] Fill in if the information has been requested and if the results of the test were negative.

Form DSMZ-BP/9 (sole page) 12/2001

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Degussa AG
Kantstr. 2
33790 Halle (Westf.)

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Degussa AG Address: Kantstr. 2 33790 Halle (Westf.)	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 15037 Date of the deposit or the transfer[1]: 2002-06-05
III. VIABILITY STATEMENT	
The viability of the microorganism identified under II above was tested on 2002-06-05 On that date, the said microorganism was (X)[3] viable ( )[3] no longer viable	
IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): U. Wirths Date: 2002-06-06

1 Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
2 In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.
3 Mark with a cross the applicable box.
4 Fill in if the information has been requested and if the results of the test were negative.

Form DSMZ-BP/9 (sole page) 12/2001

BUDAPEST INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Degussa AG
Kantstr. 2
33790 Halle (Westf.)

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: ATCC21513_17lysE::lysE	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 15037

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I. above was accompanied by:

(x) a scientific description
(x) a proposed taxonomic designation

(Mark with a cross where applicable).

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2002-06-05 (Date of the original deposit)[1].

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit)
and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b, D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): V. Weiß Date: 2002-06-06

[1] Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

Form DSMZ-BP/4 (sole page) 12/2001

BUDAPEST TRE ;RNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Degussa AG
Kantstr. 2
33790 Halle (Westf.)

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Degussa AG Address: Kantstr. 2 33790 Halle (Westf.)	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 15038 Date of the deposit or the transfer[1]: 2002-06-05

III. VIABILITY STATEMENT.

The viability of the microorganism identified under II above was tested on 2002-06-05
On that date, the said microorganism was

(X)[3] viable

( )[3] no longer viable

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): V. Weiss Date: 2002-06-06

[1] Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
[2] In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.
[3] Mark with a cross the applicable box.
[4] Fill in if the information has been requested and if the results of the test were negative.

Form DSMZ-BP/9 (sole page) 12/2001

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Degussa AG

Kantstr. 2

33790 Halle (Westf.)

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: ATCC21513_17zwal::zwal	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 15038
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I. above was accompanied by: (x) a scientific description (x) a proposed taxonomic designation (Mark with a cross where applicable).	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2002-06-05 (Date of the original deposit)[1].	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): U. Weiss Date: 2002-06-06

[1] Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Degussa AG
Kantstr. 2
33790 Halle/Künsebeck

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: DH5alphamcr/ pK18mobsacB2xlysCSma2/1	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 14244

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I. above was accompanied by:

(X) a scientific description
(X) a proposed taxonomic designation

(Mark with a cross where applicable).

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2001-04-20 (Date of the original deposit)[1].

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): U. Weiß Date: 2001-04-26

[1] Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

Form DSMZ-BP/4 (sole page) 0196

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Degussa AG
Kantstr. 2
33790 Halle/Künsebeck

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Degussa AG Kantstr. 2 Address: 33790 Halle/Künsebeck	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 14244 Date of the deposit or the transfer[1]: 2001-04-20
III. VIABILITY STATEMENT	
The viability of the microorganism identified under II above was tested on 2001-04-20 [2]. On that date, the said microorganism was (X)[3] viable ( )[3] no longer viable	
IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): V. Weiss Date: 2001-04-26

1 Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
2 In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.
3 Mark with a cross the applicable box.
4 Fill in if the information has been requested and if the results of the test were negative.

What is claimed is:

1. Coryneform bacteria which produce chemical compounds, wherein instead of the singular copy of an open reading frame (ORF), gene or allele naturally present at the particular desired site (locus), these have at least two copies of the open reading frame (ORF), gene or allele in question, preferably in tandem arrangement, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the particular site, and in that these optionally

have at least a third copy of the open reading frame (ORF), gene or allele in question at a further gene site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the further gene site.

2. Coryneform bacteria according to claim 1 which produce chemical compounds, wherein the coryneform bacteria belong to the genus Corynebacterium.

3. Coryneform bacteria of the genus Corynebacterium according to claim 2 which produce chemical compounds, wherein these belong to the species Corynebacterium glutamicum.

4. Coryneform bacteria according to claim 1 which produce chemical compounds, wherein the chemical compound is a compound chosen from the group consisting of L-amino acids, vitamins, nucleosides and nucleotides.

5. Coryneform bacteria according to claim 1 which produce chemical compounds, wherein the chemical compound is one or more L-amino acids chosen from the group consisting of L-aspartic acid, L-asparagine, L-threonine, L-serine, L-glutamic acid, L-glutamine, glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan, L-proline and L-arginine.

6. Coryneform bacteria according to claim 1 which produce chemical compounds, wherein the chemical compound is the amino acid L-lysine.

7. Coryneform bacteria which produce L-lysine, wherein instead of the singular copy of an open reading frame (ORF), gene or allele of lysine production naturally present at the particular desired site (locus), these have at least two copies of the open reading frame (ORF), gene or allele of lysine production in question, preferably in tandem arrangement, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the particular site, and in that these

optionally have at least a third copy of the open reading frame (ORF), gene or allele of lysine production in question at a further gene site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the further gene site.

8. Coryneform bacteria according to claim 7 which produce L-lysine, wherein the coryneform bacteria belong to the genus Corynebacterium.

9. Coryneform bacteria of the genus Corynebacterium according to claim 8 which produce L-lysine, wherein these belong to the species Corynebacterium glutamicum.

10. Coryneform bacteria according to claim 7 which produce L-lysine, wherein the copy of an open reading frame (ORF), gene or allele of lysine production is one or more of the open reading frames, genes or alleles chosen from the group consisting of accBC, accDA, cstA, cysD, cysE, cysH, cysK, cysN, cysQ, dapA, dapB, dapC, dapD, dapE, dapF, ddh, dps, eno, gap, gap2, gdh, gnd, lysC, $lysC^{FBR}$, lysE, msiK, opcA, oxyR, ppc, ppc^{FBR}, pgk, pknA, pknB, pknD, pknG, ppsA, ptsH, ptsI, ptsM, pyc, pyc P458S, sigC, sigD, sigE, sigH, sigM, tal, thyA, tkt, tpi, zwa1, zwf and zwf A213T.

11. Coryneform bacteria according to claim 7 which produce L-lysine, wherein the copy of an open reading frame (ORF), gene or allele of lysine production is one or more of the genes or alleles chosen from the group consisting of $lysC^{FBR}$ lysE and zwa1.

12. Coryneform bacteria according to claim 7 which produce L-lysine, wherein the copy of an open reading frame (ORF), gene or allele of lysine production is the lysE gene.

13. Coryneform bacteria according to claim 7 which produce L-lysine, wherein the copy of an open reading frame (ORF), gene or allele of lysine production is the zwa1 gene.

14. Coryneform bacteria according to claim 7 which produce L-lysine, wherein the copy of an open reading frame (ORF), gene or allele of lysine production is an $lysC^{FBR}$

allele which codes for a feed back resistant form of aspartate kinase.

15. Coryneform bacteria according to claim 14 which produce L-lysine, wherein the feed back resistant form of aspartate kinase coded by the $lysC^{FBR}$ allele contains an amino acid sequence according to SEQ ID NO:2, SEQ ID NO:2 having one or more amino acid exchanges chosen from the group consisting of A279T, A279V, S301F, T308I, S301Y, G345D, R320G, T311I and S381F.

16. Coryneform bacteria according to claim 14 which produce L-lysine, wherein the feed back resistant form of aspartate kinase coded by the $lysC^{FBR}$ allele has an amino acid sequence according to SEQ ID NO:4.

17. Coryneform bacteria according to claim 14 which produce L-lysine, wherein the coding region of the $lysC^{FBR}$ allele has the nucleotide sequence of SEQ ID NO:3.

18. Coryneform bacteria according to claim 7 which produce L-lysine, wherein the further gene site is one or more of the sites chosen from the group consisting of aecD, ccpA1, ccpA2, citA, citB, citE, fda, gluA, gluB, gluC, gluD, luxR, luxS, lysR1, lysR2, lysR3, menE, mqo, pck, pgi and poxB.

19. Coryneform bacteria according to claim 7 which produce L-lysine, wherein the further gene site is one of more of the sites chosen from the group consisting of intergenic regions of the chromosome, prophages contained in the chromosome and defective phages contained in the chromosome.

20. Processes for the preparation of one or more chemical compounds, which comprise the following steps:

a) fermentation of coryneform bacteria, which

i) instead of the singular copy of an open reading frame (ORF), gene or allele naturally present at the particular desired site (locus), have at least two copies of the said open reading frame (ORF), gene or allele, preferably in tandem arrangement, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the particular site, and which

ii) optionally have at least a third copy of the said open reading frame (ORF), gene or allele at a further gene site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the further gene site,

under conditions which allow expression of the said open reading frames (ORFs), genes or alleles,

b) concentration of the chemical compound(s) in the fermentation broth and/or in the cells of the bacteria,

c) isolation of the chemical compound(s), optionally

d) with constituents from the fermentation broth and/or the biomass to the extent of > (greater than) 0 to 100%.

21. Process according to claim 20, wherein the coryneform bacteria belong to the genus Corynebacterium.

22. Process according to claim 20, wherein the coryneform bacteria of the genus Corynebacterium belong to the species Corynebacterium glutamicum.

23. Process according to claim 20, wherein the chemical compound is a compound chosen from the group consisting of L-amino acids, vitamins, nucleosides and nucleotides.

24. Process according to claim 20, wherein the chemical compound is one or more L-amino acids chosen from the group consisting of L-aspartic acid, L-asparagine, L-threonine, L-serine, L-glutamic acid, L-glutamine, glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan, L-proline and L-arginine.

25. Process according to claim 20, wherein the chemical compound is L-lysine.

26. Process for the preparation of L-lysine, which comprises the following steps:

a) fermentation of coryneform bacteria, which

i) instead of the singular copy of an open reading frame (ORF), gene or allele of lysine production naturally present at the particular desired site (locus), have at least two copies of the said open reading frame (ORF), gene or allele, preferably in tandem arrangement, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which

imparts resistance to antibiotics being present at the particular site, and which optionally

ii) have at least a third copy of the said open reading frame (ORF), gene or allele of lysine production at a further gene site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the further gene site,

under conditions which allow expression of the said open reading frames (ORFs), genes or alleles,

b) concentration of the L-lysine in the fermentation broth and/or in the cells of the bacteria,

c) isolation of the L-lysine, optionally

d) with constituents from the fermentation broth and/or the biomass to the extent of > (greater than) 0 to 100%.

27. Process for the preparation of L-lysine according to claim 26, wherein the coryneform bacteria belong to the genus Corynebacterium.

28. Process for the preparation of L-lysine according to claim 26, wherein the coryneform bacteria of the species Corynebacterium belong to the species Corynebacterium glutamicum.

29. Process for the preparation of L-lysine according to claim 26, wherein the copy of an open reading frame (ORF), a gene or allele of lysine production is one or more of the open reading frames, genes or alleles

chosen from the group consisting of accBC, accDA, cstA, cysD, cysE, cysH, cysK, cysN, cysQ, dapA, dapB, dapC, dapD, dapE, dapF, ddh, dps, eno, gap, gap2, gdh, gnd, lysC, $lysC^{FBR}$, lysE, msiK, opcA, oxyR, ppc, ppc^{FBR}, pgk, pknA, pknB, pknD, pknG, ppsA, ptsH, ptsI, ptsM, pyc, pyc P458S, sigC, sigD, sigE, sigH, sigM, tal, thyA, tkt, tpi, zwa1, zwf and zwf A213T.

30. Process for the preparation of L-lysine according to claim 26, wherein the copy of an open reading frame (ORF), gene or allele of lysine production is one or more of the genes or alleles chosen from the group consisting of $lysC^{FBR}$, lysE and zwa1.

31. Process for the preparation of L-lysine according to claim 26, wherein the copy of an open reading frame (ORF), gene or allele of lysine production is the lysE gene.

32. Process for the preparation of L-lysine according to claim 26, wherein the copy of an open reading frame (ORF), gene or allele of lysine production is the zwa1 gene.

33. Process for the preparation of L-lysine according to claim 26, wherein the copy of an open reading frame (ORF), gene or allele of lysine production is the $lysC^{FBR}$ allele which codes for a feed back resistant form of aspartate kinase.

34. Process for the preparation of L-lysine according to claim 33, wherein the feed back resistant form of aspartate kinase coded by the $lysC^{FBR}$ allele contains an amino acid sequence according to SEQ ID NO:2, SEQ ID NO:2 having one or more amino acid exchanges chosen from the group consisting of A279T, A279V, S301F, T308I, S301Y, G345D, R320G, T311I and S381F.

35. Process for the preparation of L-lysine according to claim 33, wherein the feed back resistant form of aspartate kinase coded by the $lysC^{FBR}$ allele has an amino acid sequence according to SEQ ID NO:4.

36. Process for the preparation of L-lysine according to claim 33, wherein the coding region of the $lysC^{FBR}$ allele has the nucleotide sequence of SEQ ID NO:3.

37. Process for the preparation of L-lysine according to claim 26, wherein the further gene site is one or more of the sites chosen from the group consisting of aecD, ccpA1, ccpA2, citA, citB, citE, fda, gluA, gluB, gluC, gluD, luxR, luxS, lysR1, lysR2, lysR3, menE, mqo, pck, pgi and poxB.

38. Process for the preparation of L-lysine according to claim 26, wherein the further gene site is one of more of the sites chosen from the group consisting of intergenic regions of the chromosome, prophages contained in the chromosome and defective phages contained in the chromosome.

39. Process for the production of coryneform bacteria which produce one or more chemical compounds, wherein

a) the nucleotide sequence of a desired ORF, gene or allele, optionally including the expression and/or regulation signals, is isolated,

b) at least two copies of the nucleotide sequence of the ORF, gene or allele are arranged in a row, preferably in tandem arrangement,

c) the nucleotide sequence obtained according to b) is incorporated in a vector which does not replicate or replicates to only a limited extent in coryneform bacteria,

d) the nucleotide sequence according to b) or c) is transferred into coryneform bacteria, and

e) coryneform bacteria which have at least two copies of the desired ORF, gene or allele at the particular desired natural site instead of the singular copy of the ORF, gene or allele originally present are isolated, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remaining at the particular natural site (locus), and optionally

f) at least a third copy of the open reading frame (ORF), gene or allele in question is introduced at a further gene site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remaining at the further gene site.

40. The plasmid pK18mobsacB2xlysCSma2/1 shown in Figure 1 and deposited in the form of a pure culture of the strain E. coli DH5αmcr/pK18mobsacB2xlysCSma2/1 (=DH5alphamcr/pK18mobsacB2xlysCSma2/1) under number DSM14244.

41. The Corynebacterium glutamicum strain DSM13992lysC^{FBR}::lysC^{FBR} deposited in the form of a pure culture under number DSM15036.

42. The Corynebacterium glutamicum strain ATCC21513_17lysE::lysE deposited in the form of a pure culture under number DSM15037.

43. The Corynebacterium glutamicum strain ATCC21513_17zwa1::zwa1 deposited in the form of a pure culture under number DSM15038.

Figure 1: Plasmid pK18mobsacB2xlysCSma2/1

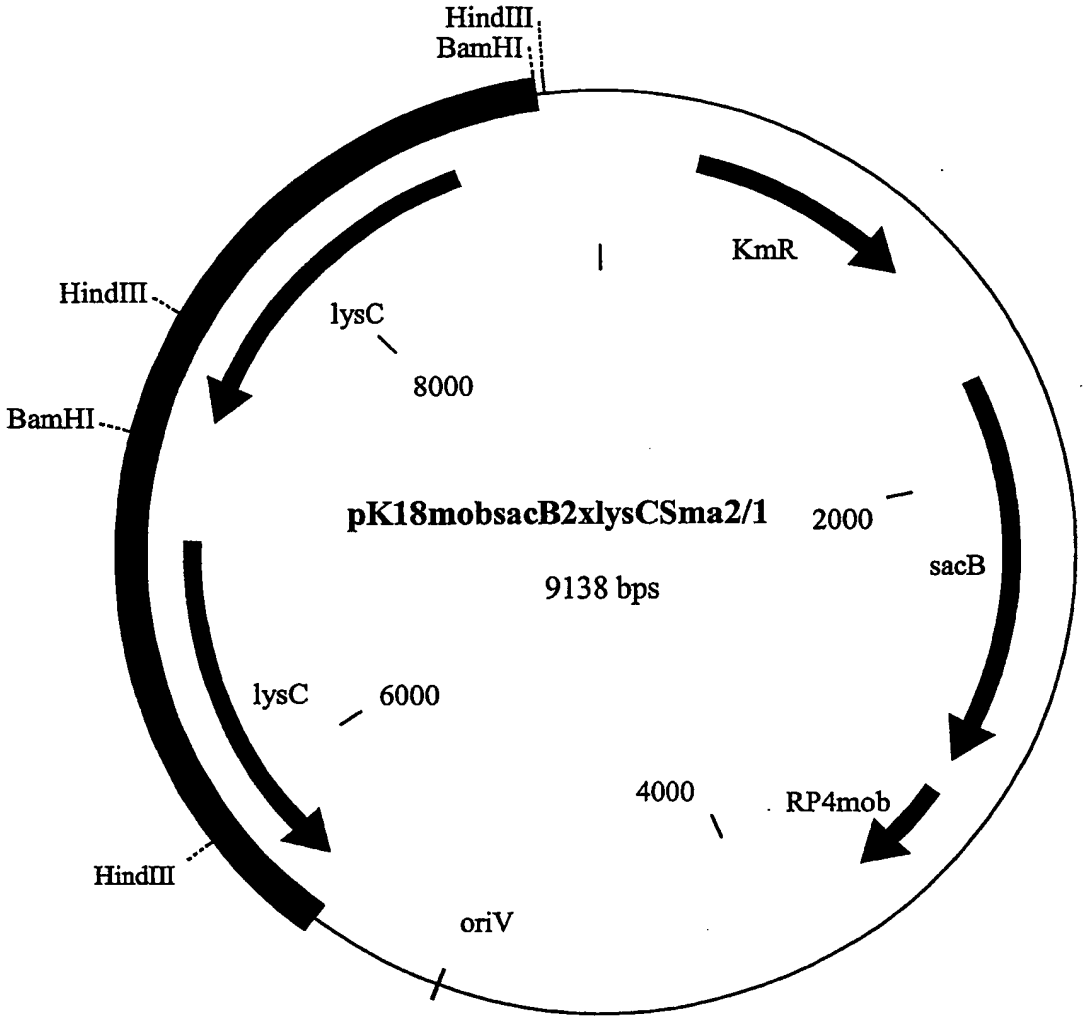


Figure 2: Plasmid pK18mobsacB2xlysESma1/1

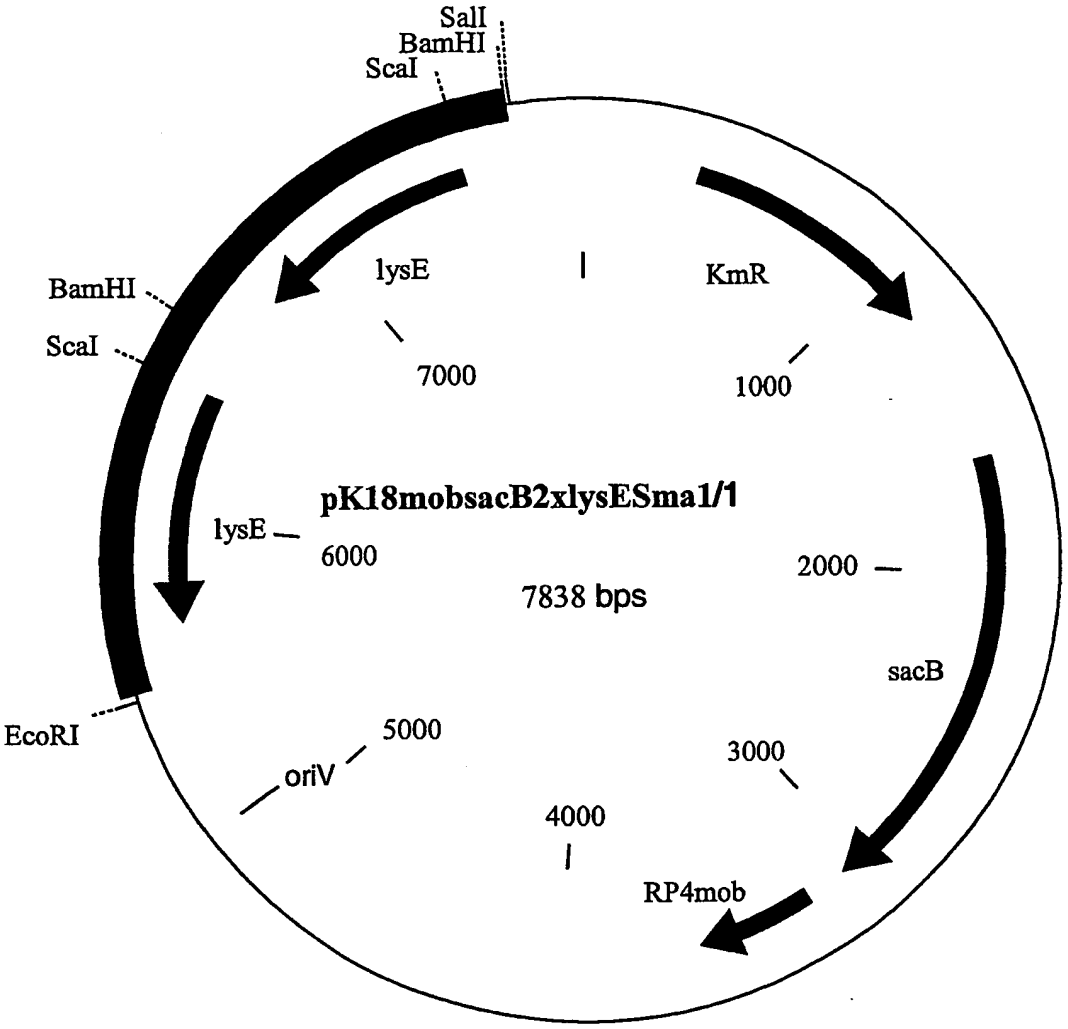
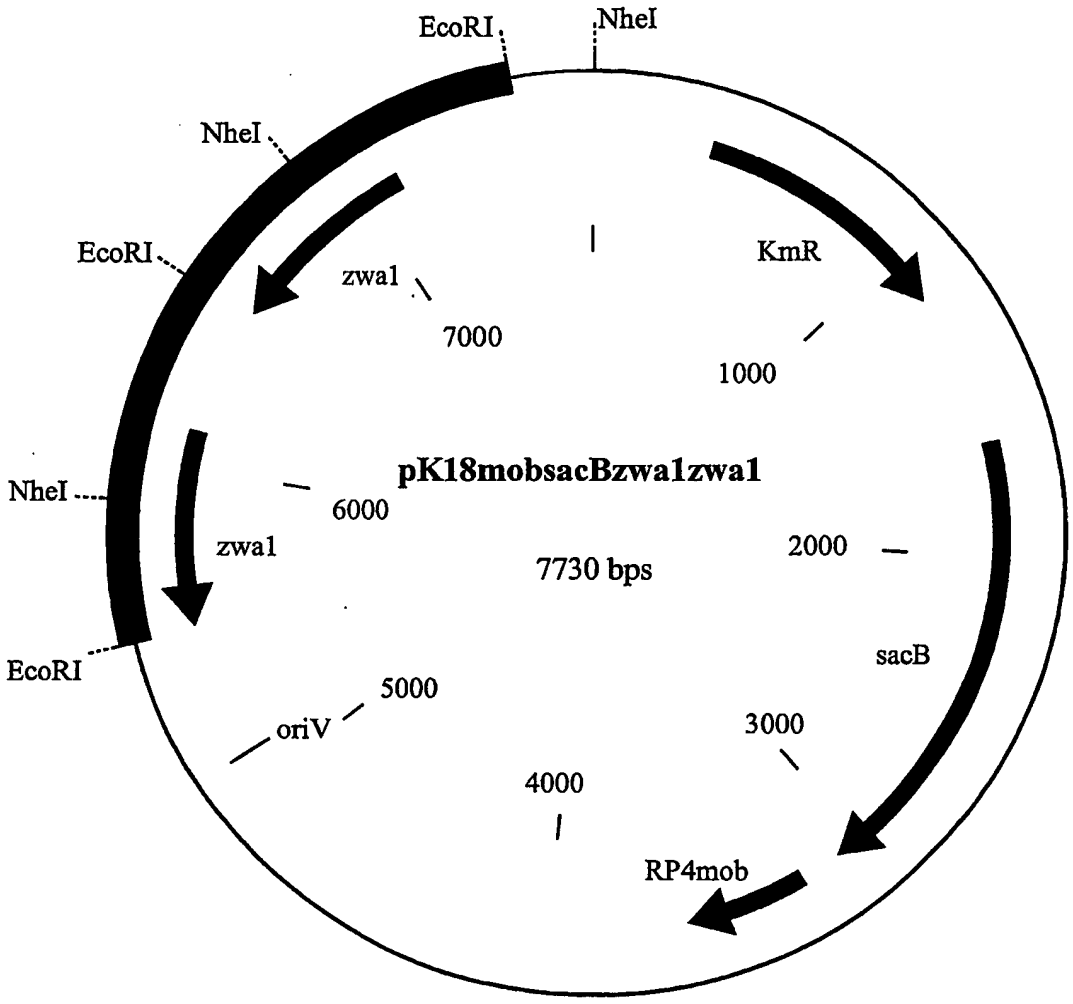


Figure 3: Plasmid pK18mobsacBzwa1zwa1



SEQUENCE LISTING

<110> Degussa AG

<120> Coryneform bacteria which produce chemical compounds II

<130> 010303 BT

<160> 14

<170> PatentIn version 3.1

<210> 1
<211> 1263
<212> DNA
<213> Corynebacterium glutamicum

<220>
<221> CDS
<222> (1)..(1263)
<223> lysC wild-type gene

<400> 1

```
gtg gcc ctg gtc gta cag aaa tat ggc ggt tcc tcg ctt gag agt gcg       48
Met Ala Leu Val Val Gln Lys Tyr Gly Gly Ser Ser Leu Glu Ser Ala
1               5                   10                  15

gaa cgc att aga aac gtc gct gaa cgg atc gtt gcc acc aag aag gct       96
Glu Arg Ile Arg Asn Val Ala Glu Arg Ile Val Ala Thr Lys Lys Ala
            20                  25                  30

gga aat gat gtc gtg gtt gtc tgc tcc gca atg gga gac acc acg gat      144
Gly Asn Asp Val Val Val Val Cys Ser Ala Met Gly Asp Thr Thr Asp
        35                  40                  45

gaa ctt cta gaa ctt gca gcg gca gtg aat ccc gtt ccg cca gct cgt      192
Glu Leu Leu Glu Leu Ala Ala Ala Val Asn Pro Val Pro Pro Ala Arg
    50                  55                  60

gaa atg gat atg ctc ctg act gct ggt gag cgt att tct aac gct ctc      240
Glu Met Asp Met Leu Leu Thr Ala Gly Glu Arg Ile Ser Asn Ala Leu
65                  70                  75                  80

gtc gcc atg gct att gag tcc ctt ggc gca gaa gcc caa tct ttc acg      288
Val Ala Met Ala Ile Glu Ser Leu Gly Ala Glu Ala Gln Ser Phe Thr
                85                  90                  95

ggc tct cag gct ggt gtg ctc acc acc gag cgc cac gga aac gca cgc      336
Gly Ser Gln Ala Gly Val Leu Thr Thr Glu Arg His Gly Asn Ala Arg
            100                 105                 110

att gtt gat gtc act cca ggt cgt gtg cgt gaa gca ctc gat gag ggc      384
Ile Val Asp Val Thr Pro Gly Arg Val Arg Glu Ala Leu Asp Glu Gly
        115                 120                 125

aag atc tgc att gtt gct ggt ttc cag ggt gtt aat aaa gaa acc cgc      432
Lys Ile Cys Ile Val Ala Gly Phe Gln Gly Val Asn Lys Glu Thr Arg
    130                 135                 140
```

```
gat gtc acc acg ttg ggt cgt ggt ggt tct gac acc act gca gtt gcg      480
Asp Val Thr Thr Leu Gly Arg Gly Gly Ser Asp Thr Thr Ala Val Ala
145                 150                 155                 160

ttg gca gct gct ttg aac gct gat gtg tgt gag att tac tcg gac gtt      528
Leu Ala Ala Ala Leu Asn Ala Asp Val Cys Glu Ile Tyr Ser Asp Val
                165                 170                 175

gac ggt gtg tat acc gct gac ccg cgc atc gtt cct aat gca cag aag      576
Asp Gly Val Tyr Thr Ala Asp Pro Arg Ile Val Pro Asn Ala Gln Lys
            180                 185                 190

ctg gaa aag ctc agc ttc gaa gaa atg ctg gaa ctt gct gct gtt ggc      624
Leu Glu Lys Leu Ser Phe Glu Glu Met Leu Glu Leu Ala Ala Val Gly
        195                 200                 205

tcc aag att ttg gtg ctg cgc agt gtt gaa tac gct cgt gca ttc aat      672
Ser Lys Ile Leu Val Leu Arg Ser Val Glu Tyr Ala Arg Ala Phe Asn
    210                 215                 220

gtg cca ctt cgc gta cgc tcg tct tat agt aat gat ccc ggc act ttg      720
Val Pro Leu Arg Val Arg Ser Ser Tyr Ser Asn Asp Pro Gly Thr Leu
225                 230                 235                 240

att gcc ggc tct atg gag gat att cct gtg gaa gaa gca gtc ctt acc      768
Ile Ala Gly Ser Met Glu Asp Ile Pro Val Glu Glu Ala Val Leu Thr
                245                 250                 255

ggt gtc gca acc gac aag tcc gaa gcc aaa gta acc gtt ctg ggt att      816
Gly Val Ala Thr Asp Lys Ser Glu Ala Lys Val Thr Val Leu Gly Ile
            260                 265                 270

tcc gat aag cca ggc gag gct gcg aag gtt ttc cgt gcg ttg gct gat      864
Ser Asp Lys Pro Gly Glu Ala Ala Lys Val Phe Arg Ala Leu Ala Asp
        275                 280                 285

gca gaa atc aac att gac atg gtt ctg cag aac gtc tct tct gta gaa      912
Ala Glu Ile Asn Ile Asp Met Val Leu Gln Asn Val Ser Ser Val Glu
    290                 295                 300

gac ggc acc acc gac atc acc ttc acc tgc cct cgt tcc gac ggc cgc      960
Asp Gly Thr Thr Asp Ile Thr Phe Thr Cys Pro Arg Ser Asp Gly Arg
305                 310                 315                 320

cgc gcg atg gag atc ttg aag aag ctt cag gtt cag ggc aac tgg acc     1008
Arg Ala Met Glu Ile Leu Lys Lys Leu Gln Val Gln Gly Asn Trp Thr
                325                 330                 335

aat gtg ctt tac gac gac cag gtc ggc aaa gtc tcc ctc gtg ggt gct     1056
Asn Val Leu Tyr Asp Asp Gln Val Gly Lys Val Ser Leu Val Gly Ala
            340                 345                 350

ggc atg aag tct cac cca ggt gtt acc gca gag ttc atg gaa gct ctg     1104
Gly Met Lys Ser His Pro Gly Val Thr Ala Glu Phe Met Glu Ala Leu
        355                 360                 365

cgc gat gtc aac gtg aac atc gaa ttg att tcc acc tct gag att cgt     1152
Arg Asp Val Asn Val Asn Ile Glu Leu Ile Ser Thr Ser Glu Ile Arg
    370                 375                 380
```

```
att tcc gtg ctg atc cgt gaa gat gat ctg gat gct gct gca cgt gca      1200
Ile Ser Val Leu Ile Arg Glu Asp Asp Leu Asp Ala Ala Ala Arg Ala
385                 390                 395                 400

ttg cat gag cag ttc cag ctg ggc ggc gaa gac gaa gcc gtc gtt tat      1248
Leu His Glu Gln Phe Gln Leu Gly Gly Glu Asp Glu Ala Val Val Tyr
                405                 410                 415

gca ggc acc gga cgc                                                  1263
Ala Gly Thr Gly Arg
            420
```

<210> 2
<211> 421
<212> PRT
<213> Corynebacterium glutamicum

<400> 2

```
Met Ala Leu Val Val Gln Lys Tyr Gly Gly Ser Ser Leu Glu Ser Ala
1               5                   10                  15

Glu Arg Ile Arg Asn Val Ala Glu Arg Ile Val Ala Thr Lys Lys Ala
            20                  25                  30

Gly Asn Asp Val Val Val Val Cys Ser Ala Met Gly Asp Thr Thr Asp
        35                  40                  45

Glu Leu Leu Glu Leu Ala Ala Ala Val Asn Pro Val Pro Pro Ala Arg
    50                  55                  60

Glu Met Asp Met Leu Leu Thr Ala Gly Glu Arg Ile Ser Asn Ala Leu
65                  70                  75                  80

Val Ala Met Ala Ile Glu Ser Leu Gly Ala Glu Ala Gln Ser Phe Thr
                85                  90                  95

Gly Ser Gln Ala Gly Val Leu Thr Thr Glu Arg His Gly Asn Ala Arg
            100                 105                 110

Ile Val Asp Val Thr Pro Gly Arg Val Arg Glu Ala Leu Asp Glu Gly
        115                 120                 125

Lys Ile Cys Ile Val Ala Gly Phe Gln Gly Val Asn Lys Glu Thr Arg
    130                 135                 140

Asp Val Thr Thr Leu Gly Arg Gly Gly Ser Asp Thr Thr Ala Val Ala
145                 150                 155                 160

Leu Ala Ala Ala Leu Asn Ala Asp Val Cys Glu Ile Tyr Ser Asp Val
                165                 170                 175

Asp Gly Val Tyr Thr Ala Asp Pro Arg Ile Val Pro Asn Ala Gln Lys
            180                 185                 190

Leu Glu Lys Leu Ser Phe Glu Glu Met Leu Glu Leu Ala Ala Val Gly
        195                 200                 205

Ser Lys Ile Leu Val Leu Arg Ser Val Glu Tyr Ala Arg Ala Phe Asn
    210                 215                 220
```

Val Pro Leu Arg Val Arg Ser Ser Tyr Ser Asn Asp Pro Gly Thr Leu
225 230 235 240

Ile Ala Gly Ser Met Glu Asp Ile Pro Val Glu Glu Ala Val Leu Thr
245 250 255

Gly Val Ala Thr Asp Lys Ser Glu Ala Lys Val Thr Val Leu Gly Ile
260 265 270

Ser Asp Lys Pro Gly Glu Ala Ala Lys Val Phe Arg Ala Leu Ala Asp
275 280 285

Ala Glu Ile Asn Ile Asp Met Val Leu Gln Asn Val Ser Ser Val Glu
290 295 300

Asp Gly Thr Thr Asp Ile Thr Phe Thr Cys Pro Arg Ser Asp Gly Arg
305 310 315 320

Arg Ala Met Glu Ile Leu Lys Lys Leu Gln Val Gln Gly Asn Trp Thr
325 330 335

Asn Val Leu Tyr Asp Asp Gln Val Gly Lys Val Ser Leu Val Gly Ala
340 345 350

Gly Met Lys Ser His Pro Gly Val Thr Ala Glu Phe Met Glu Ala Leu
355 360 365

Arg Asp Val Asn Val Asn Ile Glu Leu Ile Ser Thr Ser Glu Ile Arg
370 375 380

Ile Ser Val Leu Ile Arg Glu Asp Asp Leu Asp Ala Ala Ala Arg Ala
385 390 395 400

Leu His Glu Gln Phe Gln Leu Gly Gly Glu Asp Glu Ala Val Val Tyr
405 410 415

Ala Gly Thr Gly Arg
420

<210> 3
<211> 1263
<212> DNA
<213> Corynebacterium glutamicum

<220>
<221> CDS
<222> (1)..(1263)
<223> lysC-fbr allele lysC T311I

<400> 3
gtg gcc ctg gtc gta cag aaa tat ggc ggt tcc tcg ctt gag agt gcg 48
Met Ala Leu Val Val Gln Lys Tyr Gly Gly Ser Ser Leu Glu Ser Ala
1 5 10 15

gaa cgc att aga aac gtc gct gaa cgg atc gtt gcc acc aag aag gct 96
Glu Arg Ile Arg Asn Val Ala Glu Arg Ile Val Ala Thr Lys Lys Ala
20 25 30

gga aat gat gtc gtg gtt gtc tgc tcc gca atg gga gac acc acg gat 144
Gly Asn Asp Val Val Val Val Cys Ser Ala Met Gly Asp Thr Thr Asp
35 40 45

```
gaa ctt cta gaa ctt gca gcg gca gtg aat ccc gtt ccg cca gct cgt      192
Glu Leu Leu Glu Leu Ala Ala Ala Val Asn Pro Val Pro Pro Ala Arg
    50                  55                  60

gaa atg gat atg ctc ctg act gct ggt gag cgt att tct aac gct ctc      240
Glu Met Asp Met Leu Leu Thr Ala Gly Glu Arg Ile Ser Asn Ala Leu
65                  70                  75                  80

gtc gcc atg gct att gag tcc ctt ggc gca gaa gcc caa tct ttc acg      288
Val Ala Met Ala Ile Glu Ser Leu Gly Ala Glu Ala Gln Ser Phe Thr
                85                  90                  95

ggc tct cag gct ggt gtg ctc acc acc gag cgc cac gga aac gca cgc      336
Gly Ser Gln Ala Gly Val Leu Thr Thr Glu Arg His Gly Asn Ala Arg
            100                 105                 110

att gtt gat gtc act cca ggt cgt gtg cgt gaa gca ctc gat gag ggc      384
Ile Val Asp Val Thr Pro Gly Arg Val Arg Glu Ala Leu Asp Glu Gly
        115                 120                 125

aag atc tgc att gtt gct ggt ttc cag ggt gtt aat aaa gaa acc cgc      432
Lys Ile Cys Ile Val Ala Gly Phe Gln Gly Val Asn Lys Glu Thr Arg
    130                 135                 140

gat gtc acc acg ttg ggt cgt ggt ggt tct gac acc act gca gtt gcg      480
Asp Val Thr Thr Leu Gly Arg Gly Gly Ser Asp Thr Thr Ala Val Ala
145                 150                 155                 160

ttg gca gct gct ttg aac gct gat gtg tgt gag att tac tcg gac gtt      528
Leu Ala Ala Ala Leu Asn Ala Asp Val Cys Glu Ile Tyr Ser Asp Val
                165                 170                 175

gac ggt gtg tat acc gct gac ccg cgc atc gtt cct aat gca cag aag      576
Asp Gly Val Tyr Thr Ala Asp Pro Arg Ile Val Pro Asn Ala Gln Lys
            180                 185                 190

ctg gaa aag ctc agc ttc gaa gaa atg ctg gaa ctt gct gct gtt ggc      624
Leu Glu Lys Leu Ser Phe Glu Glu Met Leu Glu Leu Ala Ala Val Gly
        195                 200                 205

tcc aag att ttg gtg ctg cgc agt gtt gaa tac gct cgt gca ttc aat      672
Ser Lys Ile Leu Val Leu Arg Ser Val Glu Tyr Ala Arg Ala Phe Asn
    210                 215                 220

gtg cca ctt cgc gta cgc tcg tct tat agt aat gat ccc ggc act ttg      720
Val Pro Leu Arg Val Arg Ser Ser Tyr Ser Asn Asp Pro Gly Thr Leu
225                 230                 235                 240

att gcc ggc tct atg gag gat att cct gtg gaa gaa gca gtc ctt acc      768
Ile Ala Gly Ser Met Glu Asp Ile Pro Val Glu Glu Ala Val Leu Thr
                245                 250                 255

ggt gtc gca acc gac aag tcc gaa gcc aaa gta acc gtt ctg ggt att      816
Gly Val Ala Thr Asp Lys Ser Glu Ala Lys Val Thr Val Leu Gly Ile
            260                 265                 270

tcc gat aag cca ggc gag gct gcg aag gtt ttc cgt gcg ttg gct gat      864
Ser Asp Lys Pro Gly Glu Ala Ala Lys Val Phe Arg Ala Leu Ala Asp
        275                 280                 285
```

```
gca gaa atc aac att gac atg gtt ctg cag aac gtc tct tct gta gaa      912
Ala Glu Ile Asn Ile Asp Met Val Leu Gln Asn Val Ser Ser Val Glu
    290                 295                 300

gac ggc acc acc gac atc atc ttc acc tgc cct cgt tcc gac ggc cgc      960
Asp Gly Thr Thr Asp Ile Ile Phe Thr Cys Pro Arg Ser Asp Gly Arg
305                 310                 315                 320

cgc gcg atg gag atc ttg aag aag ctt cag gtt cag ggc aac tgg acc     1008
Arg Ala Met Glu Ile Leu Lys Lys Leu Gln Val Gln Gly Asn Trp Thr
                325                 330                 335

aat gtg ctt tac gac gac cag gtc ggc aaa gtc tcc ctc gtg ggt gct     1056
Asn Val Leu Tyr Asp Asp Gln Val Gly Lys Val Ser Leu Val Gly Ala
            340                 345                 350

ggc atg aag tct cac cca ggt gtt acc gca gag ttc atg gaa gct ctg     1104
Gly Met Lys Ser His Pro Gly Val Thr Ala Glu Phe Met Glu Ala Leu
        355                 360                 365

cgc gat gtc aac gtg aac atc gaa ttg att tcc acc tct gag att cgt     1152
Arg Asp Val Asn Val Asn Ile Glu Leu Ile Ser Thr Ser Glu Ile Arg
    370                 375                 380

att tcc gtg ctg atc cgt gaa gat gat ctg gat gct gct gca cgt gca     1200
Ile Ser Val Leu Ile Arg Glu Asp Asp Leu Asp Ala Ala Ala Arg Ala
385                 390                 395                 400

ttg cat gag cag ttc cag ctg ggc ggc gaa gac gaa gcc gtc gtt tat     1248
Leu His Glu Gln Phe Gln Leu Gly Gly Glu Asp Glu Ala Val Val Tyr
                405                 410                 415

gca ggc acc gga cgc                                                1263
Ala Gly Thr Gly Arg
            420
```

<210> 4
<211> 421
<212> PRT
<213> Corynebacterium glutamicum

<400> 4

```
Met Ala Leu Val Val Gln Lys Tyr Gly Gly Ser Ser Leu Glu Ser Ala
1               5                   10                  15

Glu Arg Ile Arg Asn Val Ala Glu Arg Ile Val Ala Thr Lys Lys Ala
            20                  25                  30

Gly Asn Asp Val Val Val Val Cys Ser Ala Met Gly Asp Thr Thr Asp
        35                  40                  45

Glu Leu Leu Glu Leu Ala Ala Ala Val Asn Pro Val Pro Pro Ala Arg
    50                  55                  60

Glu Met Asp Met Leu Leu Thr Ala Gly Glu Arg Ile Ser Asn Ala Leu
65                  70                  75                  80

Val Ala Met Ala Ile Glu Ser Leu Gly Ala Glu Ala Gln Ser Phe Thr
                85                  90                  95
```

Gly Ser Gln Ala Gly Val Leu Thr Thr Glu Arg His Gly Asn Ala Arg
100 105 110

Ile Val Asp Val Thr Pro Gly Arg Val Arg Glu Ala Leu Asp Glu Gly
115 120 125

Lys Ile Cys Ile Val Ala Gly Phe Gln Gly Val Asn Lys Glu Thr Arg
130 135 140

Asp Val Thr Thr Leu Gly Arg Gly Gly Ser Asp Thr Thr Ala Val Ala
145 150 155 160

Leu Ala Ala Ala Leu Asn Ala Asp Val Cys Glu Ile Tyr Ser Asp Val
165 170 175

Asp Gly Val Tyr Thr Ala Asp Pro Arg Ile Val Pro Asn Ala Gln Lys
180 185 190

Leu Glu Lys Leu Ser Phe Glu Glu Met Leu Glu Leu Ala Ala Val Gly
195 200 205

Ser Lys Ile Leu Val Leu Arg Ser Val Glu Tyr Ala Arg Ala Phe Asn
210 215 220

Val Pro Leu Arg Val Arg Ser Ser Tyr Ser Asn Asp Pro Gly Thr Leu
225 230 235 240

Ile Ala Gly Ser Met Glu Asp Ile Pro Val Glu Glu Ala Val Leu Thr
245 250 255

Gly Val Ala Thr Asp Lys Ser Glu Ala Lys Val Thr Val Leu Gly Ile
260 265 270

Ser Asp Lys Pro Gly Glu Ala Ala Lys Val Phe Arg Ala Leu Ala Asp
275 280 285

Ala Glu Ile Asn Ile Asp Met Val Leu Gln Asn Val Ser Ser Val Glu
290 295 300

Asp Gly Thr Thr Asp Ile Ile Phe Thr Cys Pro Arg Ser Asp Gly Arg
305 310 315 320

Arg Ala Met Glu Ile Leu Lys Lys Leu Gln Val Gln Gly Asn Trp Thr
325 330 335

Asn Val Leu Tyr Asp Asp Gln Val Gly Lys Val Ser Leu Val Gly Ala
340 345 350

Gly Met Lys Ser His Pro Gly Val Thr Ala Glu Phe Met Glu Ala Leu
355 360 365

Arg Asp Val Asn Val Asn Ile Glu Leu Ile Ser Thr Ser Glu Ile Arg
370 375 380

Ile Ser Val Leu Ile Arg Glu Asp Asp Leu Asp Ala Ala Ala Arg Ala
385 390 395 400

Leu His Glu Gln Phe Gln Leu Gly Gly Glu Asp Glu Ala Val Val Tyr
405 410 415

Ala Gly Thr Gly Arg
420

<210> 5
<211> 20
<212> DNA
<213> Corynebacterium glutamicum

<220>
<221> misc_feature
<222> (1)..(20)
<223> Primer lysCK1

<400> 5
tcggtgtcat cagagcattg 20

<210> 6
<211> 20
<212> DNA
<213> Corynebacterium glutamicum

<220>
<221> misc_feature
<222> (1)..(20)
<223> Primer lysCK2

<400> 6
tcggttgcct gagtaatgtc 20

<210> 7
<211> 20
<212> DNA
<213> Corynebacterium glutamicum

<220>
<221> misc_feature
<222> (1)..(20)
<223> LC-lysC1-fbr

<400> 7
aaccgttctg ggtatttccg 20

<210> 8
<211> 20
<212> DNA
<213> Corynebacterium glutamicum

<220>
<221> misc_feature
<222> (1)..(20)
<223> LC-lysC2-fbr

<400> 8
tccatgaact ctgcggtaac 20

<210> 9
<211> 21
<212> DNA
<213> Corynebacterium glutamicum

<220>
<221> misc_feature
<222> (1)..(21)
<223> oligonucleotide lysC311-C

<400> 9
gcaggtgaag atgatgtcgg t 21

<210> 10
<211> 35
<212> DNA
<213> Corynebacterium glutamicum

<220>
<221> misc_feature
<222> (1)..(35)
<223> Oligonukleotid lysC311-A

<400> 10
tcaagatctc catcgcgcgg cggccgtcgg aacga 35

<210> 11
<211> 20
<212> DNA
<213> Corynebacterium glutamicum

<220>
<221> misc_feature
<222> (1)..(20)
<223> Primer lysEK-1

<400> 11
tgcttgcaca aggacttcac 20

<210> 12
<211> 20
<212> DNA
<213> Corynebacterium glutamicum

<220>
<221> misc_feature
<222> (1)..(20)
<223> Primer lysEK-2

<400> 12
tatggtccgc aagctcaatg 20

<210> 13
<211> 20
<212> DNA
<213> Corynebacterium glutamicum

<220>
<221> misc_feature
<222> (1)..(20)
<223> Primer zwal-A2

<400> 13
cacttgtcct caccactttc 20

<210> 14
<211> 20
<212> DNA
<213> Corynebacterium glutamicum

<220>
<221> misc_feature
<222> (1)..(20)
<223> Primer zwa1-E1

<400> 14
ttctactggg cgtactttcg 20

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
20 February 2003 (20.02.2003)

PCT

(10) International Publication Number
WO 03/014330 A3

(51) International Patent Classification[7]: C12P 13/08
// C12R 1/15

(21) International Application Number: PCT/EP02/08465

(22) International Filing Date: 30 July 2002 (30.07.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/309,877 6 August 2001 (06.08.2001) US

(71) Applicant *(for all designated States except US)*: **DEGUSSA AG** [DE/DE]; Bennigsenplatz 1, 40474 Düsseldorf (DE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **BATHE, Brigitte** [DE/DE]; Twieten 1, 33154 Salzkotten (DE). **KREUTZER, Caroline** [DE/DE]; Poststrasse 16, 49326 Melle (DE). **MÖCKEL, Bettina** [DE/DE]; Benrodestrasse 35, 40597 Düsseldorf (DE). **THIERBACH, Georg** [DE/DE]; Gunststrasse 21, 33613 Bielefeld (DE).

(74) Common Representative: **DEGUSSA AG**; Intellectual Property Management, Patents and Trademarks, Location Hanau, P. O. Box 13 45, 63403 Hanau (DE).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW, ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)*
— *of inventorship (Rule 4.17(iv)) for US only*

Published:
— *with international search report*

(88) Date of publication of the international search report:
18 December 2003

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/014330 A3

(54) Title: CORYNEFORM BACTERIA WHICH PRODUCE CHEMICAL COMPOUNDS II

(57) Abstract: The invention relates to coryneform bacteria, which instead of the singular copy of an open reading frame (ORF), gene or allele naturally present at the particular desired site (locus), have at least two copies of the open reading frame (ORF), gene or allele in question, preferably in tandem arrangement, and optionally at least a third copy of the open reading frame (ORF), gene or allele in question at a further gene site, and processes for the preparation of chemical compounds by fermentation of these bacteria.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 02/08465

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12P13/08 //C12R1/15

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12P C12R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, PAJ, EPO-Internal, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 111 062 A (DEGUSSA) 27 June 2001 (2001-06-27) page 5, paragraph 30 -page 6, paragraph 35 ---	1-43
X	EP 1 108 790 A (KYOWA HAKKO KOGYO KK) 20 June 2001 (2001-06-20) The copy only comprises the relevant parts of the document. page 24, paragraph 228; figure 1 ---	1-43
X	US 6 200 785 B1 (KREUTZER CAROLINE ET AL) 13 March 2001 (2001-03-13) column 2, line 45 -column 4, line 6 ---	1-43
X	WO 99 18228 A (PETERS WENDISCH PETRA ;EIKMANNS BERND (DE); SAHM HERMANN (DE); KER) 15 April 1999 (1999-04-15) page 7, last paragraph ---	1-43
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 June 2003	30/06/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Griesinger, I

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 02/08465

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SCHAEFER A ET AL: "SMALL MOBILIZABLE MULTI-PURPOSE CLONING VECTORS DERIVED FROM THE ESCHERICHIA COLI PLASMIDS PK18 AND PK19: SELECTION OF DEFINED DELETIONS IN THE CHROMOSOME OF CORYNEBACTERIUM GLUTAMICUM" GENE, ELSEVIER BIOMEDICAL PRESS. AMSTERDAM, NL, vol. 145, no. 145, 1994, pages 69-73, XP001093898 ISSN: 0378-1119 abstract ---	1-43
A	EIKMANNS B J ET AL: "MOLECULAR ASPECTS OF LYSINE, THREONINE, AND ISOLEUCINE BIOSYNTHESIS IN CORYNEBACTERIUM GLUTAMICUM" ANTONIE VAN LEEUWENHOEK, DORDRECHT, NL, vol. 64, no. 2, 1993, pages 145-163, XP000918559 page 151, left-hand column, paragraph 1 page 157, left-hand column, paragraph 2 ---	1-43
P,X	WO 02 22632 A (DEGUSSA) 21 March 2002 (2002-03-21) page 11, line 17 -page 15, line 13 ------	1-43

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 02/08465

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-43 (each claim partially)
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-43 (each claim partially)

The present set of claims refers to products and methods defined by 1. the absence of features, namely the absence of sequences which enable episomal replication and the absence of sequences enabling transposition and the absence of sequences providing antibiotics resistance and by presence of a vague feature, namely the presence of at least two copies of any kind of an ORF at a "particular desired site". The claims cover all products and methods having these characteristics, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such products and methods. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). The feature of an ORF "at a particular desired site" is completely unclear. The additional features are unclear, since they are defined as a result, which should not be achieved. This lack of clarity renders a meaningful search over the whole of the claimed scope impossible.

Furthermore, the present set of claims refers to any "chemical compound", which can be produced by Corynebacterium. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds claimed, namely for lysine (see Table 1 and Examples 1-3) and in particular for the use of the genes lysC, lysE and zwal for the production of lysine. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible.

Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely 1. the principle of inserting two copies of a gene, which naturally occurs in Corynebacterium glutamicum, into the bacterial chromosome of Corynebacterium glutamicum wherein said two copies are only separated from each other by a "small" number of nucleotides, which do not encode a protein and wherein said bacterial chromosome does not contain any other vector sequences and 2. the specific embodiments relating to the insertion of two copies of the genes lysC, lysE or zwal as disclosed in the Examples 1-3. Said limitations of the search are based on the Examples 1-3.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 02/08465

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
EP 1111062	A	27-06-2001	DE	19959328 A1	13-06-2001
			AU	7201800 A	14-06-2001
			BR	0005804 A	20-11-2001
			CN	1304998 A	25-07-2001
			EP	1111062 A1	27-06-2001
			HU	0004880 A2	28-10-2002
			JP	2001197893 A	24-07-2001
			PL	344386 A1	18-06-2001
			SK	18342000 A3	03-12-2001
			US	2002127663 A1	12-09-2002
EP 1108790	A	20-06-2001	EP	1108790 A2	20-06-2001
			JP	2002191370 A	09-07-2002
			US	2002197605 A1	26-12-2002
US 6200785	B1	13-03-2001	DE	19931317 A1	11-01-2001
			AU	4509900 A	11-01-2001
			BR	0002655 A	05-06-2001
			CA	2310870 A1	07-01-2001
			CN	1280184 A	17-01-2001
			EP	1067192 A1	10-01-2001
			HU	0002585 A2	28-09-2002
			JP	2001061485 A	13-03-2001
			SK	10142000 A3	09-04-2001
			US	2002086371 A1	04-07-2002
WO 9918228	A	15-04-1999	DE	19831609 A1	15-04-1999
			AU	741038 B2	22-11-2001
			AU	1148299 A	27-04-1999
			BR	9813021 A	15-08-2000
			CA	2305577 A1	15-04-1999
			CN	1275167 T	29-11-2000
			WO	9918228 A2	15-04-1999
			EP	1015621 A2	05-07-2000
			HU	0004381 A2	28-03-2001
			JP	2002508921 T	26-03-2002
			SK	4812000 A3	12-09-2000
			ZA	9809014 A	03-04-2000
WO 0222632	A	21-03-2002	DE	10120094 A1	28-03-2002
			AU	9553901 A	26-03-2002
			WO	0222632 A2	21-03-2002
			US	2002039766 A1	04-04-2002